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INTERNATIONAL APPLICATION PUBLISHED UNDER

WO 9606175A2

(51) International Patent Classification 6 : C12N 15/55, A01N 63/02, A01H 5/00, C12N 1/21		A2	(11) International Publication Number: WO 9606175 (43) International Publication Date: 29 February 1996 (29.02.96)
(21) International Application Number: PCT/US95/10284 (22) International Filing Date: 11 August 1995 (11.08.95) (30) Priority Data: 08/289,595 12 August 1994 (12.08.94) US 08/484,815 7 June 1995 (07.06.95) US (71) Applicant: PIONEER HI-BRED INTERNATIONAL, INC. [US/US]; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US). (72) Inventors: DUVICK, Jonathan; 1707 38th Street, Des Moines, IA 50310 (US). ROOD, Tracy, A.; 4333 Allison Avenue, Des Moines, IA 50310 (US). (74) Agents: BOBROWICZ, Donna et al.; 700 Capital Square, 400 Locust Street, Des Moines, IA 50309 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG).  Published Without international search report and to be republished upon receipt of that report.	
(54) Title: FUMONISIN-DETOXIFYING ENZYMES			
(57) Abstract  Methods for identifying organisms capable of degrading fumonisin. Fumonisin can be incorporated into culture medium for selection of organisms resistant to fumonisin and/or capable of growing on fumonisin as a sole carbon source. Using this method, several organisms have been identified. These organisms can be used to isolate the enzyme and the gene responsible for conferring fumonisin-resistance. The gene can be cloned and inserted into a suitable expression vector so that the protein can be further characterized. Additionally, the DNA encoding for fumonisin-resistance can be used to transform plant cells normally susceptible to <i>Fusarium</i> or other toxin-producing fungus infection. Plants can be regenerated from the transformed plant cells. In this way, a transgenic plant can be produced with the capability of degrading fumonisin, as well as with the capability of producing the degrading enzymes. Methods for detoxification in grain processing and in animal feed and rumen microbes are also disclosed.			
<div>ACTAGTGGAT CATTGCAFTG GCTGGCGGAC TGGCGCGCG ATAGTCGTTG 1</div> <div>CGATGGTGGC GAGAATAGGC GTGGGAGTGG GAGGAGATGG AAGATGGGGG 51</div> <div>CGAGGAGTAT GTGTGGCGGA CGTTTGGGAC GCTTCTGCAI TGGCTTGGCT 101</div> <div>CGATGGTGGC CGGTGACTCT AGGGGAGGAC TGGCGCGCG GGGCGCGGAC 151</div> <div>CGATGGTGGC TTTGGGCTCC GAGGAGGAGG TCTGGGCGAG GTTCAGGGAC 201</div> <div>GTGGGCGGAC GTGGGAGGAC TTTGGGCGGA TGGCTTGGAC AGGGCGCGCG 251</div> <div>GTGGGCGGAC TGGGTTGGAA GGGCGCGGAA CAGGGCGGCG CTTGGGCGGG 301</div> <div>GTGGGCGGAC GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA CTTGGGCGGG 351</div> <div>CGGTGGCGCG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 401</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 451</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 501</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 551</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 601</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 651</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 701</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 751</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 801</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 851</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 901</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 951</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1001</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1051</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1101</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1151</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1201</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1251</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1301</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1351</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1401</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1451</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1501</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1551</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1601</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1651</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1701</div> <div>AGGTATGGG GGGCGGCGAA TTTGGGCGGA TTTGGGCGGA TTTGGGCGGG 1751</div>			

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## FUMONISIN-DETOXIFYING ENZYMES.

This is a continuation-in-part of prior copending U.S. application Serial No. 08/289,595, filed August 12, 1994.

5    **Technical Field**

The present invention relates generally to the detection and isolation of fumonisin resistant organisms and to compositions and methods for the *in vivo* detoxification or degradation of fumonisin. This method has broad application in agricultural biotechnology and crop agriculture and in the improvement of food grain quality.

10   **Background of the Invention**

Fungal diseases are common problems in crop agriculture. Many strides have been made against plant diseases as exemplified by the use of hybrid plants, pesticides and improved agricultural practices. However, as any grower or home gardener can attest, the problems of fungal plant disease continue to cause difficulties in plant cultivation. Thus, there is a  
15    continuing need for new methods and materials for solving the problems caused by fungal diseases of plants. These problems can be met through a variety of approaches. For example, the infectious organisms can be controlled through the use of agents that are selectively biocidal for the pathogens. Another method is interference with the mechanism by which the pathogen invades the host crop plant. Yet another method, in the case of pathogens that cause  
20    crop losses, is interference with the mechanism by which the pathogen causes injury to the host crop plant. Still another method, in the case of pathogens that produce toxins that are undesirable to mammals or other animals that feed on the crop plants, is interference with toxin production, storage, or activity. This invention falls into the latter two categories.

Since their discovery and structural elucidation in 1988 (Bezuidenhout S, Gelderblom  
25    W, Gorst-Allman C, Horak R, Marasas W, Spiteller B, Vleggaar R (1988) "Structure elucidation of the fumonisins, mycotoxins from *Fusarium moniliforme*." *Journal Chem Soc, Chem Commun* 1988: 743-745), fumonisins have been recognized as a potentially serious problem in maize-fed livestock. They are linked to several animal toxicoses including leukoencephalomalacia (Marasas WFO, Kellerman TS, Gelderblom WCA, Coetzer JAW,  
30    Thiel P (1988) "Leukoencephalomalacia in a horse induced by fumonisin B-1 isolated from *Fusarium moniliforme*." *Onderstepoort Journal of Veterinary Research* 55: 197-204; Wilson TM, Ledet AE, Owens DL, Rice LG, Nelson HA (1990) "Experimental liver

disease in ponies associated with the ingestion of a corn-based ration naturally contaminated with fumonisin B<sub>1</sub>," **American Association of Veterinary Laboratory Diagnosticians: Abstracts 33rd Annual Meeting**, Denver, Colorado, October 7-9, 1990., Madison, Wisconsin, USA) and porcine pulmonary edema (Colvin BM, Harrison LR (1992)

5 "Fumonisin-Induced Pulmonary Edema and Hydrothorax in Swine." *Mycopathologia* 117: 79-82). and are also suspected carcinogens (Geary W (1971) *Coord Chem Rev* 7: 81; Gelderblom WCA, Kriek NPJ, Marasas WFO, Thiel PG (1991) "Toxicity and Carcinogenicity of the *Fusarium*-*Moniliforme* Metabolite, Fumonisin-B<sub>1</sub>, in Rats." *Carcinogenesis* 12: 1247-1251; Gelderblom WCA, Semple E, Marasas WFO, Farber E

10 (1992) "The Cancer-Initiating Potential of the Fumonisin-B Mycotoxins." *Carcinogenesis* 13: 433-437). *Fusarium* isolates in section *Liseola* produce fumonisins in culture at levels from 2 to >4000 ppm (Leslie J, Plattner R, Desjardins A, Klittich C (1992) "Fumonisin B<sub>1</sub> production by strains from different mating populations of *Gibberella fujikoro* (*Fusarium* section *Liseola*)." *Phytopathology* 82: 341-345). Isolates from maize (predominantly mating

15 population A ) are among the highest producers (Leslie *et al.*, *supra*). Fumonisin levels detected in field-grown maize have fluctuated widely depending on location and growing season, but both preharvest and postharvest surveys of field maize have indicated that the potential for high levels of fumonisins exists (Murphy PA, Rice LG, Ross PF (1993) "Fumonisin-B<sub>1</sub>, Fumonisin-B<sub>2</sub>, and Fumonisin-B<sub>3</sub> content of Iowa, Wisconsin, and Illinois

20 corn and corn screenings." *J Agr Food Chem* 41: 263-266). Surveys of food and feed products have also detected fumonisin (Holcomb M, Thompson HC Jr., Hankins LJ (1993) "Analysis of fumonisin B-1 in rodent feed by gradient elution HPLC using precolumn derivation with FMOC and fluorescence detection." *J Agr Food Chem* 41: 764-767; Hopmans EC, Murphy PA (1993) "Detection of Fumonisin-B(1), Fumonisin-B(2), and

25 Fumonisin-B(3) and hydrolyzed Fumonisin-B(1) in Corn-Containing foods." *J Agr Food Chem* 41: 1655-1658; Sydenham EW, Shephard GS, Thiel PG, Marasas WFO, Stockenstrom S (1991) "Fumonisin Contamination of Commercial Corn-Based Human Foodstuffs." *J Agr Food Chem* 39: 2014-2018). The etiology of *Fusarium* ear mold is poorly understood, although physical damage to the ear and certain environmental conditions

30 can contribute to its occurrence (Nelson PE (1992) "Taxonomy and Biology of *Fusarium moniliforme*." *Mycopathologia* 117: 29-36). *Fusarium* can be isolated from most field grown maize, even when no visible mold is present. The relationship between seedling infection and

stalk and ear diseases caused by *Fusarium* is not clear. Genetic resistance to visible kernel mold has been identified (Gendloff E, Rossman E, Casale W, Isleib T, Hart P (1986) "Components of resistance to *Fusarium* ear rot in field corn." **Phytopathology** 76: 684-688; Holley RN, Hamilton PB, Goodman MM (1989) "Evaluation of tropical maize germplasm for resistance to kernel colonization by *Fusarium moniliforme*." **Plant Dis** 73: 578-580), but the relationship to visible mold to fumonisin production has yet to be elucidated.

Fumonisin have been shown in *in vitro* mammalian cell studies to inhibit sphingolipid biosynthesis through inhibition of the enzyme sphinganine acyl transferase (Norred WP, Wang E, Yoo H, Riley RT, Merrill AH (1992) "In vitro toxicology of fumonisins and the mechanistic implications." **Mycopathologia** 117: 73-78; Wang E, Norred W, Bacon C, Riley R, Merrill A Jr. (1991) "Inhibition of sphingolipid biosynthesis by fumonisins: implications for diseases associated with *Fusarium moniliforme*." **J Biol Chem** 266: 14486; Yoo HS, Norred WP, Wang E, Merrill AH, Riley RT (1992) "Fumonisin Inhibition of *de Novo* Sphingolipid Biosynthesis and Cytotoxicity Are Correlated in LLC-PK1 Cells." **Toxicol Appl Pharmacol** 114: 9-15), resulting in the accumulation of the precursor sphinganine. It is likely that inhibition of this pathway accounts for at least some of fumonisin's toxicity, and support for this comes from measures of sphinganine:sphingosine ratios in animals fed purified fumonisin (Wang E, Ross PF, Wilson TM, Riley RT, Merrill AH (1992) "Increases in Serum Sphingosine and Sphinganine and Decreases in Complex Sphingolipids in Ponies Given Feed Containing Fumonisin, Mycotoxins Produced by *Fusarium moniliforme*." **J Nutr** 122: 1706-1716). Fumonisin also affect plant cell growth (Abbas HK, Boyette CD (1992) "Phytotoxicity of fumonisin B<sub>1</sub> on weed and crop species." **Weed Technol** 6: 548-552; Vanasch MAJ, Rijkenberg FHJ, Coutinho TA (1992) "Phytotoxicity of fumonisin B<sub>1</sub>, moniliformin, and t-2 toxin to corn callus cultures." **Phytopathology** 82: 1330-1332; Vesonder RF, Peterson RE, Labeda D, Abbas HK (1992) "Comparative phytotoxicity of the fumonisins, AAL-Toxin and yeast sphingolipids in *Lemna minor* L. (Duckweed)." **Arch Environ Contam Toxicol** 23: 464-467). Kuti *et al.* "Effect of fumonisin B<sub>1</sub> on virulence of *Fusarium* species isolated from tomato plants." (Abstract, Annual Meeting American Phytopathological Society, Memphis, TN: APS Press 1993) reported on the ability of exogenously added fumonisins to accelerate disease development and increase sporulation of *Fusarium moniliforme* and *F. oxysporum* on tomato.

The toxicity of fumonisins and their potential widespread occurrence in food and makes it imperative to find detoxification or elimination strategies to remove the compound from the food chain.

#### Disclosure of the Invention

5       The present invention is based on the discovery of organisms with the ability to degrade the mycotoxin fumonisin. In a search for a biological means of detoxifying fumonisins, we have isolated from field-grown maize kernels several dematiaceous hyphomycetes capable of growing on fumonisin B<sub>1</sub> or B<sub>2</sub> (FB<sub>1</sub> or FB<sub>2</sub>) as a sole carbon source, degrading it partially or completely in the process. One species, identified as *Exophiala*  
10 *spinifera*, a "black yeast", was recovered from maize seed from diverse locations in the southeastern and south central U.S. A related species, *Rhinoctadiella atrovirens*, was isolated from seed originating in both Iowa and Georgia. We also isolated a bacterium, believed to be a *Xanthomonas* or *Sphingomonas* species, designated isolate 2412.1, from a field-grown maize stalk sample from Johnston, Iowa. This bacterium also showed growth on FB<sub>1</sub> as a sole  
15 carbon source, and since its taxonomy is not certain we have deposited the strain with the American Type Culture Collection (ATCC) and it is referred to herein by its ATCC deposit number, 55552. We have also deposited enzyme-active strains of *Exophiala spinifera* (ATCC 74269) and *Rhinoctadiella atrovirens* (ATCC 74270).

All isolates showed the capability to degrade FB<sub>1</sub> in liquid culture. By "degrade" is  
20 simply meant that the enzyme is capable of using fumonisin as a substrate and converting it to a different chemical structure. However, our studies indicate that the resulting compounds are less toxic than the fumonisins themselves.

Overall, only 16 of 70 independent seed samples tested yielded degraders. However, several *E. spinifera* isolates, collected outside the U.S. from non-maize sources, were also  
25 found to metabolize fumonisins. Representative isolates of other *Exophiala* species tested (*E. jeanselmi*, *E. salmonis*, *E. piscifera*) did not degrade fumonisins, nor did non-maize *Rhinoctadiella* isolates, including *R. atrovirens* and *R. anceps*, nor fungi associated with ear molds including *Fusarium moniliforme*, *F. graminearum*, *Aspergillus flavus* and *Diplodia maydis*. Fumonisin-metabolizing black yeasts were found to possess an inducible hydrolase  
30 activity that cleaves the tricarballylate esters of FB<sub>1</sub>, as monitored by C<sub>18</sub>-thin layer chromatography (TLC) and fluorescence detection of amines. The identity of the resulting amino alcohol compound, designated AP<sub>1</sub>, was verified by FAB-mass spectroscopy. The latter

compound has utility as a chemical indicator of fumonisin metabolism. *E. spinifera* cultures further metabolized AP<sub>1</sub> to compounds of unknown identity that were not detectable by amine reagents on TLC. In sealed culture chambers, *E. spinifera* grown on <sup>14</sup>C FB<sub>1</sub> as a sole carbon source, released <sup>14</sup>CO<sub>2</sub> as detected in 1N KOH-saturated filler paper strips, totaling percent of added label in 48 hours. Heat-killed cultures similarly incubated did not release appreciable <sup>14</sup>CO<sub>2</sub>. Thus, at least a portion of the fumonisin is fully metabolized by this fungus. Crude, cell-free culture filtrates of the *E. spinifera* isolate designated 2141.10 contained a heat-labile, protease-sensitive hydrolase activity attributed to an enzyme tentatively characterized as an esterase with specificity for tricarballylate esters of fumonisins and similar molecules such as AAL-toxin from *Alternaria alternata lycopersici*. This purified esterase is believed to be a new chemical entity, since no commercially available esterases tested were able to hydrolyze the tricarballylate esters of FB<sub>1</sub>, suggesting a novel enzyme specificity produced by these fungi. Cell-free extracts of *E. spinifera* isolate 2141.10 also contain an AP<sub>1</sub> catabolase capable of converting AP<sub>1</sub> to a compound lacking a free amine group, possibly an aldehyde. These enzymes and genes coding for these enzymes, being involved in fumonisin degradation, have utility in detoxification of maize seed pre- or post-harvest.

#### **Brief Description of the Drawings**

FIGURE 1 is the nucleotide sequence including the open reading frame coding for the bacterial esterase gene (bases 94 to 1683).

FIGURE 2 is a hypothetical amino acid sequence of the polypeptide encoded by bases 94 to 1683 of the nucleotide sequence of FIGURE 1. Residues 1-38 represent the putative signal sequence. The polypeptide including the signal sequence has a calculated molecular weight of 55,026.68 (529 residues), with a calculated pI of 8.70. The polypeptide without the putative signal sequence has a calculated molecular weight of 51,495.63 (491 residues), with a calculated pI of 8.19.

#### **Detailed Description of the Invention**

This invention provides newly discovered enzymes capable of degrading and detoxifying fumonisins, produced by fermentation of one or more of *Exophiala spinifera*, ATCC 74269, *Rhinochadiella atrovirens*, ATCC 74270, or the bacterium of ATCC 55552.

The invention further comprises methods for making enzymes that are capable of detoxifying fumonisins, comprising the step of growing one or more of *Exophiala spinifera*, ATCC 74269, *Rhinochadiella atrovirens*, ATCC 74270, or the bacterium ATCC 55552 in the presence of a

fumonisin or the metabolite produced by action of the enzyme on a fumonisins. This invention further provides methods of detoxifying fumonisins, comprising the step of reacting them with an enzyme derived from *Exophiala spinifera*, ATCC 74269, *Rhinocladiella atrovirens*, ATCC 74270, or the bacterium of ATCC 55552.

5 We have isolated and sequenced the gene that codes for the fumonisins-degrading enzyme from one of these organisms and provide the amino acid sequence of the enzyme here. It is known that genes encoding desired proteins can be identified, isolated, cloned and expressed in transgenic organisms, including several important crop plants. One commonly used method of gene transfer in plants involves the use of a disarmed form of the Ti plasmid of the soil  
10 bacterium *Agrobacterium tumefaciens*. *A. tumefaciens* is a plant pathogen that causes crown-gall tumors in infected plants. Large plasmids, termed Ti- or tumor-inducing plasmids, are responsible for the oncogenicity of the bacterium as well as for the transfer of foreign DNA to the plant. Similarly, *A. rhizogenes* contains Ri- or root-inducing plasmids that induce root growth. Both plasmid types include a *vir* or virulence region that must be functional in order  
15 to transform wild-type cells to tumor cells.

Transformation results in the integration of another plasmid portion, termed the T- or transfer-DNA, into the nuclear genome of the transformed cells. Ri and Ti plasmids can be manipulated to allow insertion of foreign DNA, encoding a desired protein, into the T-DNA region. The foreign DNA can be transferred either via a vector bearing both the *vir* gene and  
20 the foreign gene or by a binary vector system consisting of two plasmids, one containing the *vir* gene and the other carrying the foreign gene. See, e.g., U.S. Pat. No. 4,658,082. Transformed plant cells can then be regenerated to produce varieties bearing the inserted gene. The production of transgenic, fumonisins-resistant plants will provide a useful and novel approach for the control of *Fusarium*-induced plant diseases.

25 This invention also provides a mechanism for selection of transformants: growth of plant cells in the presence of a *Fusarium* or its mycotoxin favors the survival of plant cells that have been transformed to express the coding sequence that codes for the enzyme of this invention and degrade the toxin. Thus, the coding sequence that codes for the enzyme of this invention can itself be used as a selectable marker, or as a scorable marker by measuring  
30 formation of the amino alcohol metabolite.

Another embodiment of the present invention is directed to a DNA construct comprising an expression cassette comprised of:

- a) a DNA coding sequence for a polypeptide capable of degrading fumonisins; and



b) control sequences that are operably linked to the coding sequence whereby the coding sequence can be transcribed and translated in a host cell, and at least one of the DNA coding sequences or control sequences is heterologous to the host cell.

5 Preferred embodiments of the subject invention include a host cell stably transformed by a DNA construct as described above; and a method of producing a polypeptide of a recombinant gene comprising:

a) providing a population of these host cells; and

b) growing the population of cells under conditions whereby the polypeptide encoded  
10 by the coding sequence of the expression cassette is expressed.

In yet another embodiment, the present invention is directed to a transgenic plant capable of degrading fumonisin. In another embodiment, the transgenic plant is a maize plant capable of degrading fumonisin.

Another embodiment of the subject invention comprises a method of conferring  
15 fumonisin-resistance to a plant substantially without such resistance comprising transferring to the plant an expressible gene encoding a polypeptide capable of degrading fumonisin.

Thus, DNA encoding a protein able to inactivate fumonisin can be isolated and cloned in an appropriate vector and inserted into an organism normally sensitive to the *Fusarium* or its toxin. Organisms expressing the gene can be easily identified by their ability to degrade  
20 fumonisin. The protein capable of degrading fumonisin can be isolated and characterized using techniques well known in the art. Furthermore, the gene imparting fumonisin-resistance can be transferred into a suitable plasmid, such as into the T-DNA region of the Ti or Ri plasmid of the soil bacteria *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, respectively. Plant tissue can be inoculated with the transformed bacteria. Additionally, plant  
25 tissues which have been co-cultivated with *Agrobacterium* spp. can be incubated in the presence of fumonisin to select for fumonisin-degrading transgenic plants, i.e., the gene for fumonisin degradation can serve as a selectable marker. Thus, the inoculated tissue is regenerated to produce fumonisin-degrading transgenic plants.

30 Additionally, the present invention relates to ruminal microorganisms that have been genetically engineered with the genes imparting fumonisin resistance. These engineered ruminal microorganisms can then be added to feed for consumption by animals susceptible to fumonisin and structurally related mycotoxins.

### Industrial Applicability

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of botany, microbiology, tissue culture, molecular biology, chemistry, biochemistry and recombinant DNA technology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., J. H. Langenheim and K. V. Thimann, *Botany: Plant Biology and Its Relation to Human Affairs* (1982) John Wiley; *Cell Culture and Somatic Cell Genetics of Plants*, Vol. 1 (I. K. Vasil, ed. 1984); R. V. Stanier, J. L. Ingraham, M. L. Wheelis, and P. R. Painter, *The Microbial World*, (1986) 5th Ed., Prentice-Hall; O. D. Dhringra and J. B. Sinclair, *Basic Plant Pathology Methods*, (1985) CRC Press; Maniatis, Fritsch & Sambrook, *Molecular Cloning: A Laboratory Manual* (1982); *DNA Cloning*, Vols. I and II (D. N. Glover ed. 1985); *Oligonucleotide Synthesis* (M. J. Gait ed. 1984); *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); and the series *Methods in Enzymology* (S. Colowick and N. Kaplan, eds., Academic Press, Inc.).

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

By "microbe" is meant any microorganism (including both eucaryotic and procaryotic microorganisms), such as fungi, yeasts, bacteria, actinomycetes, algae and protozoa, as well as other unicellular structures capable of growth in culture.

A "fumonisin-producing microbe" is any microbe capable of producing the mycotoxin fumonisin or analogs thereof. Such microbes are generally members of the fungal genus *Fusarium*, as well as recombinantly derived organisms which have been genetically altered to enable them to produce fumonisin or analogues thereof.

By "degrading fumonisin" is meant any modification to the fumonisin molecule which causes a decrease or loss in its toxic activity. Such a change can comprise cleavage of any of the various bonds, oxidation, reduction, the addition or deletion of a chemical moiety, or any other change that affects the activity of the molecule. In a preferred embodiment, the modification includes hydrolysis of the ester linkage in the molecule as a first step.

Furthermore, chemically altered fumonisin can be isolated from cultures of microbes that produce an enzyme of this invention, such as by growing the organisms on media containing radioactively-labeled fumonisin, tracing the label, and isolating the degraded toxin for further study. The degraded fumonisin can be compared to the active compound for its

phytotoxicity or mammalian toxicity in known sensitive species, such as porcines and equines. Such toxicity assays are known in the art. For example, in plants a whole leaf bioassay can be used in which solutions of the active and inactive compound are applied to the leaves of sensitive plants. The leaves may be treated *in situ* or, alternatively, excised leaves may be used. The relative toxicity of the compounds can be estimated by grading the ensuing damage to the plant tissues and by measuring the size of lesions formed within a given time period. Other known assays can be performed at the cellular level, employing standard tissue culture methodologies e.g., using cell suspension cultures.

By "structurally related mycotoxin" is meant any mycotoxin having a chemical structure related to a fumonisin such as fumonisin B1, for example AAL toxin, fumonisin B2, fumonisin B3, fumonisin B4, fumonisin C1, fumonisin A1 and A2, and their analogs, as well as other mycotoxins having similar chemical structures that would be expected to be detoxified by activity of the fumonisin degradative enzymes elaborated by *Exophiala spinifera*, ATCC 74269, *Rhinochadiella atrovirens*, ATCC 74270, or the bacteria of ATCC 55552.

By "harvested grain" is meant any form of grain which has been somehow removed from the environment in which it was grown. For example, harvested grain could constitute ear corn, or corn kernels removed from the ear, or cut wheat stalks, or barley or rice kernels, or the like. Harvested grain may be in storage or may be being processed. "Processed grain" is grain that has been through some form of processing and will be used in the production of food for human consumption or will be used as animal feed.

By "transgenic plant" is meant any plant or plant cell that has become transformed by the introduction, stable and heritable incorporation, into the subject plant or plant cell, of foreign DNA, i.e. DNA encoding for a protein not normally found within that plant species.

"Plantlet" refers to a plant sufficiently developed to have a shoot and a root that is asexually reproduced by cell culture.

"Explant" refers to a section or piece of tissue from any part of a plant for culturing.

By "hormone" is meant any plant growth regulator that affects the growth or differentiation of plant cells. Such hormones include cytokinins, auxins, and gibberellins, as well as other substances capable of affecting plant cells.

The term "callus" and its plural "calli", refer to an unorganized group of cells formed in response to cutting, severing, or other injury inflicted on plant tissue. Excised pieces of plant tissue and isolated cells can be induced to form callus under the appropriate culture conditions.

Callus can be maintained in culture for a considerable time by transferring or subculturing parts of the callus to fresh medium at regular intervals. The transfer of callus to liquid medium leads to dispersion of the tissue and the formation of a plant cell suspension culture. Callus can be induced to undergo organized development to form shoots and roots.

5 "Embryoid" refers to a structure similar in appearance to a plant zygotic embryo.

"Somatic hybrid" and "somatic hybridization" refers generally to stable combination of cellular material, be it protoplast/protoplast or protoplast/cytoplast combinations, and includes cybrids and cybridization.

10 A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication in vivo; i.e., capable of replication under its own control.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

15 As used herein, the term "nucleotide sequence" means a DNA or RNA molecule or sequence, and can include, for example, a cDNA, genomic DNA, or a synthetic DNA sequence, a structural gene or a fragment thereof, or an mRNA sequence that encodes an active or functional polypeptide.

20 A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the  
25 coding sequence.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bound at its 3' terminus by the translation start codon (ATG) of a coding sequence and extends  
30 upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase.

Eucaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Procarvotic promoters contain Shine-Dalgarno sequences.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

A coding sequence is "operably linked to" or "under the control of" control sequences in a cell when RNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA, which is then translated into the polypeptide encoded by the coding sequence.

A "host cell" is a cell which has been transformed, or is capable of undergoing transformation, by an exogenous DNA sequence.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced inside the cell membrane. Exogenous DNA may or may not be integrated into (covalently linked to) chromosomal DNA making up the genome of the transformed cell. In procaryotes and yeasts for example, the exogenous DNA may be maintained on an episomal element, such as a plasmid. With respect to eucaryotic cells, a stably transformed cell is one in which the exogenous DNA has become integrated into the chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eucaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the exogenous DNA.

A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth in vitro for many generations.

Two DNA, RNA or polypeptide sequences are "substantially homologous" when at least about 85% (preferably at least about 90%, and most preferably at least about 95%) of the nucleotides or amino acids match over a defined length of the molecule. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, e.g., Maniatis et al., supra; DNA Cloning, Vols. I & II, supra; Nucleic Acid Hybridization, supra.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within or attached to another DNA molecule that is not found in association with the other molecule in nature. Thus, when the heterologous region encodes a bacterial gene, the gene will usually be flanked by DNA that does not flank the bacterial gene in the genome of the source  
5 bacterium. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene). "Heterologous" DNA also refers to DNA not found within the host cell in nature. Allelic variation or naturally occurring mutational events do not give rise to a heterologous region of DNA, as these terms are used herein.

10 The term "polypeptide" as used herein is used in its broadest sense, i.e., any polymer of amino acids (dipeptide or greater) linked through peptide bonds. Thus, the term "polypeptide" includes proteins, oligopeptides, protein fragments, analogues, muteins, fusion proteins and the like. The term also encompasses amino acid polymers as described above that include additional non-amino acid moieties. Thus, the term "polypeptide" includes glycoproteins,  
15 lipoproteins, phosphoproteins, metalloproteins, nucleoproteins, as well as other conjugated proteins. The term "polypeptide" contemplates polypeptides as defined above that are recombinantly produced, isolated from an appropriate source, or synthesized.

This invention can be better understood by reference to the following non-limiting examples. It will be appreciated by those skilled in the art that other embodiments of the  
20 invention may be practiced without departing from the spirit and the scope of the invention as herein disclosed and claimed.

#### Example 1

**Chemicals and reagents.** All chemicals were reagent grade or better unless otherwise indicated. Fumonisin B<sub>1</sub> and B<sub>2</sub> were obtained from Sigma Chemical Co. Partially purified  
25 fumonisins (eluate from C8 column) were obtained from Dr. Pat Murphy (Iowa State University). AAL-toxin (TA isomer) was a gift of Dr. David Gilchrist (University of California-Davis).

**Plant tissue samples.** Mature, field-grown maize seed was obtained from maize breeding locations of Pioneer Hi-Bred International, Inc. in the Southeast, Midwest and South  
30 Central regions of the U.S. Seed was stored at room temperature in individual packets.

**Fungal and bacterial isolates.** *Exophiala* and *Rhinoctadiella* isolates from maize were isolated as described below. Other isolates were obtained from Dr. C.J. Wang (Syracuse,

NY), Dr. Michael McGinnis (Case Western Reserve University, Cleveland, OH), and from the American Type Culture Collection (Bethesda, MD). *Fusarium graminearum* [*Gibberella zeae* (Schw.) Petsch], *Diplodia maydis*, and *Fusarium moniliforme* Sheld., were obtained from the microbial culture collection of Pioneer Hi-Bred International, Inc.. *Aspergillus flavus* (Schw.) Petsch, isolate CP22, was obtained from Don Sumner at the University of Georgia (Tifton, GA). *Xanthomonas* sp. 2412.1 was isolated from maize stalk tissue as described below.

**Isolation methods.** Individual kernels, either intact or split in two with a sterile razor blade, were rinsed for 1 hr in 5 ml sterile water with agitation. From 1 to 5  $\mu$ l of the rinse fluid was added to 100  $\mu$ L of sterile, carbon-free mineral salts medium + FB<sub>1</sub> (MS-FB<sub>1</sub>) (1 g/liter NH<sub>3</sub>SO<sub>4</sub>, 1 g/liter K<sub>2</sub>HPO<sub>4</sub>, 1 g/liter NaCl, 0.2 g/liter MgSO<sub>4</sub>·7H<sub>2</sub>O, pH 7.0) containing FB<sub>1</sub> (Sigma Chemical Co.) at 0.5 to 1.0 mg/ml). The pH of the medium was approx. 6.0 after addition of FB<sub>1</sub>. After 1 to 2 weeks incubation at 28°C in the dark, serial 10-fold dilutions were made in sterile dH<sub>2</sub>O, and aliquots were plated onto 1.2% Bacto-agar containing 0.1% yeast extract, 1% Bacto-peptone and 0.1% dextrose (YPD agar). Fungal and bacterial colonies that appeared on agar were transferred onto fresh plates and individual colonies were evaluated for fumonisin metabolizing ability by inoculating them into fresh MS-FB<sub>1</sub>. Loss of fumonisin from the medium was monitored periodically by spotting 0.5 to 1 microliter aliquots of culture supernatant on C<sub>18</sub> silica gel plates that were then air-dried and developed as described below (see Analysis of fumonisins and metabolism products).

Direct isolation of black yeasts from seed was accomplished by plating 100 microliters of seed wash fluid onto YPD or Sabouraud agar augmented with cycloheximide (500 mg/liter) and chloramphenicol (50 mg/liter). Plates were incubated at room temperature for 7-14 days, and individual pigmented colonies that arose were counted and cultured for analysis of fumonisin-degrading ability as described above.

For stalk isolations, mature stalk samples 0.5 x 0.5 x 2 cm were taken from Southern-type maize inbreds grown in Johnston, Iowa by Pioneer Hi-Bred International, Inc., a seed company, in 1993. One-inch sections of the center (pith) or the outside of non-surface-sterilized stalk were cut and placed in 10 ml. sterile water in a small, sterilized tube. The tubes were shaken for 1 hour, and then 2  $\mu$ l of washate were withdrawn and used to inoculate 100  $\mu$ l of MS-FB<sub>1</sub> in a microtiter plate. Subsequent steps were as above.

**Analysis of fumonisins and metabolism products.** Analytical thin-layer chromatography was carried out on 100% silanized C<sub>18</sub> silica plates (Sigma™ #T-7020; 10 x 10 cm; 0.1 mm thick) by a modification of the published method of Rottinghaus. Sample lanes were pre-wet with methanol to facilitate sample application. After application of from 0.1 to 2  
 5  $\mu$ l of aqueous sample, the plates were air-dried and developed in MeOH:4% KCl (3:2) or MeOH:0.2 M KOH (3:2) and then sprayed successively with 0.1 M sodium borate (pH 9.5) and fluorescamine (0.4 mg/ml in acetonitrile). Plates were air-dried and viewed under long wave UV.

**Alkaline hydrolysis of FB<sub>1</sub> to AP<sub>1</sub>.** FB<sub>1</sub> or crude fumonisin C<sub>8</sub> material was  
 10 suspended in water at 10-100 mg/ml and added to an equal volume of 4 N NaOH in a screw-cap tube. The tube was sealed and incubated at 60°C for 1 hr. The hydrolysate was cooled to RT and mixed with an equal volume of ethyl acetate, centrifuged at 1000 RCF for 5 minute and the organic (upper) layer recovered. The pooled ethyl acetate layers from two successive extractions were dried under N<sub>2</sub> and resuspended in dH<sub>2</sub>O. The resulting material (the  
 15 aminopentol of FB<sub>1</sub> or "AP<sub>1</sub>") was analyzed by TLC.

Tables 1 and 2 illustrate the results of efforts to isolate a fumonisin-degrading enzyme from a wide assortment of sources. As is noted, *E. spinifera* isolates from maize seed from various locations were always able to produce a fumonisin-degrading enzyme when grown on fumonisin as a sole carbon source (Table 1), as were *E. spinifera* isolates from other sources  
 20 from around the world (Table 2). Some samples of *Rhinoctadiella atrovirens* from maize seed were also able to produce this enzyme (Table 1). Other species of *Exophiala* and other sources and species of *Rhinoctadiella* were routinely unable to produce the enzyme, even when isolated from plant-related sources (Table 2).

Table 1: *Dematiaceae* fungi isolated from maize seed that degrade fumonisin

Isolate#	Species	Location of origin	Isolated from	Appearance <sup>1</sup>	Modification of substrates	
					FB <sub>1</sub>	AP <sub>1</sub>
2369.E7	<i>Exophiala spinifera</i>	Tifton, GA	Maize seed (3293)	clean	+	+
2369.G5	<i>Exophiala spinifera</i>	Tifton, GA	Maize seed (3379)	clean	+	+
2174.A4	<i>Exophiala spinifera</i>	Tifton, GA	Maize seed (inbred)	moldy	+	+



Isolate#	Species	Location of origin	Isolated from	Appearance <sup>1</sup>	Modification of substrates	
					FB <sub>1</sub>	AP <sub>1</sub>
2369.F7	<i>Exophiala spinifera</i>	Winterville, NC	Maize seed (3170)	moldy	+	+
2369.H9	<i>Exophiala spinifera</i>	Winterville, NC	Maize seed (3379)	moldy	+	+
2141.10	<i>Exophiala spinifera</i>	Winterville, NC	Maize seed (unk)	moldy	+	+
2174.C6	<i>Rhino-cladiella atrovirens</i>	Winterville, NC	Maize seed (unk)	moldy	+	+
2170.2	<i>Exophiala spinifera</i>	Winterville, NC	Maize seed (inbred)	moldy	+	+
2174.A4	<i>Exophiala spinifera</i>	Union City, TN	Maize seed (inbred)	moldy?	+	+
2219.H5	<i>Exophiala spinifera</i>	Union City, TN	Maize seed (inbred)	moldy	+	+
2363.1	<i>Exophiala spinifera</i>	Weslaco, TX	Maize seed (inbred)	moldy	+	+
2363.3	<i>Exophiala spinifera</i>	Weslaco, TX	Maize seed (inbred)	moldy	+	+
2363.3	<i>Exophiala spinifera</i>	Weslaco, TX	Maize seed (inbred)	moldy	+	+
2363.8	<i>Exophiala spinifera</i>	Weslaco, TX	Maize seed (inbred)	moldy	+	+
2363.10	<i>Exophiala spinifera</i>	Weslaco, TX	Maize seed (inbred)	moldy	nt	
2369.F11	<i>Rhino-cladiella atrovirens</i>	Johnston, LA	Maize seed (inbred)	clean	+	+

<sup>1</sup> "moldy" implies visible discoloration of kernel pericarp, cracking or splitting; "clean" implies no visible signs of infection on the kernel

<sup>2</sup> Evaluated by TLC analysis of culture supernatants as described herein

nt = not tested

Table 2: Other fungal isolates tested for degradation of fumonisin B1 in liquid culture

Isolate	Species	Source	Location of Origin	Isolated from	Modification of substrates	
					FB <sub>1</sub>	AP <sub>1</sub>
-Black Yeast Fungi -						
26089	<i>Exophiala spinifera</i>	ATCC	Uruguay	Palm trunk	+	+
26090	<i>Exophiala spinifera</i>	ATCC	Uruguay	Palm tree fruit	+	+
26091	<i>Exophiala spinifera</i>	ATCC	Uruguay	Bird's nest	+	+
26092	<i>Exophiala spinifera</i>	ATCC	Uruguay	Bird's nest	+	+
48173	<i>Exophiala spinifera</i>	ATCC		Nasal Granuloma	+	+
56567	<i>Exophiala spinifera</i>	ATCC		?	+	+
18218	<i>Exophiala spinifera</i>	ATCC		Nasal Granuloma	+	+
58092	<i>Exophiala spinifera</i>	ATCC		Human	+	+
66775 <sup>m</sup>	<i>Exophiala monileae</i>	ATCC			-	nt
32288	<i>Exophiala salmonis</i>	ATCC	Unknown	Leaf Litter	-	nt
26438	<i>Exophiala pisciphila</i>	ATCC	Australia	Wheat rhizosphere	-	nt
26272	<i>Exophiala jeanselmi</i>	ATCC	Canada	Activated sludge	-	nt
P-154	<i>Rhinocladiella atrovirens</i>	C.J. Wang	Chester, NJ	Southern pine pole	-	nt
P-330	<i>Rhinocladiella atrovirens</i>	C.J. Wang	Binghamton, NY	Southern pine pole	-	nt
P-646	<i>Rhinocladiella atrovirens</i>	C.J. Wang	Virginia	Southern pine pole	-	nt
P-1492	<i>Rhinocladiella atrovirens</i>	C.J. Wang	Chester, NJ	Southern pine pole	-	nt

Isolate	Species	Source	Location of Origin	Isolated from	Modification of substrates	
					FB <sub>1</sub>	AP <sub>1</sub>
ED-43	<i>Rhinoctadiella atrovirens</i>	C.J. Wang	Unknown	Douglas-fir pole	-	nt
ED-124	<i>Rhinoctadiella atrovirens</i>	C.J. Wang	Unknown	Douglas-fir pole	-	nt
28220	<i>Rhinoctadiella anceps</i>	ATCC	Maryland	Grass	-	nt
-Ear mold fungi -						
FMO001	<i>Fusarium moniliforme</i>	PHI	Unknown	Maize	-	nt
FGR001	<i>Fusarium graminearum</i>	PHI	Unknown	Maize	-	nt
CP22	<i>Aspergillus flavus</i>	PHI	Unknown	Maize	-	nt
DMA001	<i>Diplodia maydis</i>	PHI	Unknown	Maize	-	nt

\*Tested both with FB<sub>1</sub> and as a sole carbon source and with FB<sub>1</sub> amended with 1% sucrose.

Table 3: Frequency of isolation of fumonisin-degrading black yeast isolates from maize seed

Location of origin <sup>a</sup>	# samples tested	# samples positive	% containing FB <sub>1</sub> -degrading black yeast	Species identified
Weslaco, TX	6	6	75.0	<i>Exophiala spinifera</i>
Winterville, NC	19	4	47.5	<i>Exophiala spinifera</i> , <i>Rhinoctadiella atrovirens</i>
Tifton, GA	8	3	37.5	<i>Exophiala spinifera</i>
Union City, TN <sup>2</sup>	7	2	28.2	<i>Exophiala spinifera</i>
Johnston, LA	7	1	14.3	<i>Rhinoctadiella atrovirens</i>
Shelbyville, IL	3	0	0	none
Macomb, IL	4	0	0	-
Champaign, IL	3	0	0	-
Yale, IN	3	0	0	-
California	8	0	0	-
Total	70	16	22.8	

Organisms can be screened for their ability to degrade fumonisin using the present methods. In this way, plant, soil, marine and fresh water samples can be screened and organisms isolated therefrom that are able to degrade fumonisin. Alternatively, already isolated microbial strains that are suspected of possessing this capability can be screened.

- 5 Putative fumonisin-resistant bacteria include bacteria associated with plant species susceptible to *Fusarium* infection. For instance, bacteria associated with *Fusarium*-infected tomato and pepper as well as other susceptible plant species, might be expected to degrade fumonisin. Furthermore, members of bacterial genera known to be versatile in their catabolism of complex organic molecules, such as members of the genus *Pseudomonas*, might degrade  
10 fumonisin.

Generally, media used to culture the above microbes will contain a known amount of fumonisin, i.e. from 0.1 to 3 mg of fumonisin per ml of media, more usually from .25 to 2 mg per ml of media, and preferably from 0.5 to 1 mg of fumonisin per ml of media.

- A further study was performed to determine if colony morphology could be used to  
15 determine which strains of these species would produce a fumonisin-degrading enzyme. The results as shown in Table 4 indicated that *E. spinifera* and *R. atrovirens* colonies having different morphologies could nevertheless produce the fumonisin-degrading enzyme.

**Table 4: Black yeasts recovered from a single kernel by direct plating seed washates onto YPD + cycloheximide + chloramphenicol<sup>1</sup>**

Isolate	Colony Type on YPD agar	Species	# colonies	# FB <sub>1</sub> degr
2403.5	Light brown, shiny	<i>Exophiala spinifera</i>	33	33
2403.25	Dark brown, shiny	<i>Exophiala spinifera</i>	1	1
2403.12	Brown, velvety	<i>Rhinochadiella</i> <i>atrovirens</i>	4	4
2403.2	Grey, velvety	<i>Rhinochadiella</i> <i>atrovirens</i>	1	1
Totals			39	39

- 20 <sup>1</sup> Kernel source: Tifton, Georgia. Seed was split, washed in 5 ml sterile water and then 100 ul was plated onto YPD agar containing cycloheximide (500 mg/L) and chloramphenicol (50 mg/L).

From these results it was concluded that growth on fumonisin as the sole carbon source is the most reliable indicator of the ability to produce the fumonisin-degrading esterase.

The esterase isolated from *E. spinifera* was then subjected to other treatments, including proteases, to determine whether and how the enzyme would function in various environments. The results are indicated in Table 5.

Table 5: Effect of various treatments on modification of FB<sub>1</sub>

Treatment	Conditions	FB <sub>1</sub> Hydrolase activity <sup>a</sup>
Control	16 hr, 37° C, pH 5.2	+++
Boiling water bath	100° C, 30 min, pH 5.2	-
Protease K	0.01 mg/ml, 16 hr, 37° C, pH 5.2	+
Pronase E	0.01 mg/ml, 16 hr, 37° C, pH 5.2	++
Chymotrypsin	0.01 mg/ml, 16 hr, 37° C, pH 5.2	++
Trypsin	0.01 mg/ml, 16 hr, 37° C, pH 5.2	+++
EDTA	50 mM	++
DTT	25 mM	+++
Ca <sup>2+</sup>	50 mM	+++
Mg <sup>2+</sup>	50 mM	+++
PMSF	10 mM	+++

<sup>a</sup> 10-fold concentrated, 11 to 15 day culture filtrates treated as described and then incubated with FB<sub>1</sub> (0.5 mg/ml final conc) overnight at 37°C. Analysis by C<sub>18</sub> TLC/fluorescamine spray following overnight incubation at 37°C with 1 mg/ml fumonisin

- = no hydrolysis  
 ± = trace amount of hydrolysis  
 + = incomplete hydrolysis  
 ++ = incomplete hydrolysis  
 +++ = complete hydrolysis

Next, the pH range of activity of the fumonisin esterase was evaluated by measuring fumonisin degradation in the presence of citrate and citrate-phosphate buffers at varying pH levels. Results are shown in Table 6. From this, it was concluded that the pH range of the enzyme was quite wide, and that the enzyme would function at the internal pH of plants and plant cells.

Table 6: Effect of buffer pH on hydrolysis of fumonisin B<sub>1</sub>  
by *E. spinifera* culture filtrate

Buffer	pH	FB <sub>1</sub> Hydrolase activity*
0.1 M citrate	3.0	+++
0.1 M citrate-phosphate	4.0	+++
0.1 M citrate-phosphate	5.0	++
0.1 M citrate-phosphate	6.0	++
0.1 M phosphate	7.0	±
0.1 M phosphate	8.0	-

\* reactions were carried out at 37°C overnight and then assayed by TLC

- 5 \* Analysis by C<sub>18</sub> TLC/fluorescamine spray following overnight incubation at 37°C with 1 mg/ml fumonisin.

- = no hydrolysis  
 ± = trace amount of hydrolysis  
 + = incomplete hydrolysis  
 10 ++ = incomplete hydrolysis  
 +++ = complete hydrolysis..

The fumonisin esterase isolated from *E. spinifera* and *R. atrovirens* was compared with other known esterases from various sources as supplied by commercial vendors. The results shown in Table 7 indicate that the fumonisin esterase is a unique enzyme that is highly specific  
 15 in its activity and does not have a generalized esterase activity comparable to that of any of the known enzymes tested.

Table 7: Hydrolysis of fumonisin B<sub>1</sub> by commercial esterases and hydrolases

Enzyme	Code	Source, purity	Units/m g prot.	Units per rxn	Assay pH	FB <sub>1</sub> hydrolysis
Esterase, nonspecific	EC 3.1.1.1	Rabbit	100		8.0	-
Esterase, nonspecific	EC 3.1.1.1	Porcine liver	200		7.5	-
Lipase	EC 3.1.1.3	Candida cylindrica	35		7.7	-
Cholinesterase, butyryl	EC 3.1.1.8	Horse serum, highly purified	500	15	8.0	-

Enzyme	Code	Source, purity	Units/m g prot.	Units per rxn	Assay pH	FB <sub>1</sub> hydrolysis
Cholinesterase, acetyl	EC 3.1.1.7	Bovine, partially pure	0.33	0.15	8.0	-
Cholesterol esterase	EC 3.1.1.13	Bovine, partially pure	0.5	0.15	8.0	-
Cholesterol esterase	EC 3.1.1.13	Porcine, partially pure		0.15	8.0	-
Cholesterol esterase	EC 3.1.1.13	<i>Pseudomonas</i> <i>fluorescens</i>	12	1.5	7.0	-
Cholesterol esterase	EC 3.1.1.13	<i>Pseudomonas</i> sp.	200	15	7.0	±
Acetylcholinesterase	EC 3.1.1.6	Orange Peel partially pure	4	0.15	6.5	-
Pectinesterase	EC 3.1.1.11	Orange Peel, partially pure	100	1.5	7.5	-
Pectinase	EC 3.2.1.15	<i>Rhizopus</i> Crude	0.5	1.5	4.0	-
Pectinase	EC 3.2.1.15	<i>Aspergillus</i> Partially pure	5	0.1	4.0	-
Fumonisin esterase	?	<i>Exophiala</i> <i>spinifera</i> , crude	unk	unk	5.2	+++

\* Analysis by C<sub>18</sub>-TLC/fluorescamine spray following overnight incubation at 37° C with 1 mg/ml fumonisin.

- = no hydrolysis  
 ± = trace amount of hydrolysis  
 + = incomplete hydrolysis  
 ++ = incomplete hydrolysis  
 +++ = complete hydrolysis

The enzyme of this invention was evaluated for inducibility by growing an *Exophiala* culture on various carbon sources of varying degrees of structural similarity to fumonisin. The results, shown in Table 8, illustrate that both the original form of fumonisin and its metabolite are capable of inducing enzyme production, but that inducibility of the enzyme is also quite specific.

Table 8: Ability of various carbon sources to support growth and/or induction of FB<sub>1</sub> hydrolytic activity

<i>Exophiala</i> culture activity			
Carbon source	Concentration	Growth	FB <sub>1</sub> hydrolase activity
FB <sub>1</sub>	0.1%	+	+
Alkaline hydrolyzed FB <sub>1</sub> (API)	0.1%	+	+
Na+ Tricarballylate	0.1%	±	-
Sphingosine	0.1%	-	-
Phytosphingosine	0.1%	-	-
Na+ Citrate	0.1%	+	-
Sucrose	0.1%	+	-
Glucose	0.1%	+	-

The ability of the fumonisin esterase to cleave other organic carboxylesters was also evaluated in comparison to its ability to hydrolyse fumonisin. The results, shown in Table 8, also illustrate that the enzyme hydrolyzed the tricarballylates of other related aminoalcohols such as FB<sub>2</sub> and AAL toxin.

Table 9: Hydrolysis of organic carboxylesters by *Exophiala* crude concentrated culture filtrate

Substrate	Conditions	Assay method	Hydrolysis by <i>Exophiala</i> culture filtrate
FB <sub>1</sub>	pH 5.2, 37° C, 1 hr	C <sub>18</sub> TLC: fluorescamine	+
FB <sub>2</sub>	pH 5.2, 37° C, 1 hr	C <sub>18</sub> TLC: fluorescamine	+
AAL-toxin	pH 5.2, 37° C, 1 hr	C <sub>18</sub> TLC: fluorescamine	+

#### Enzyme activity of culture filtrate and mycelium. *Exophiala spinifera* isolate

2141.10 was grown on YPD agar for 1 week, and conidia were harvested, suspended in sterile water, and used at 105 conidia per ml to inoculate sterile Fries mineral salts medium containing 1 mg/ml purified FB<sub>1</sub> (Sigma Chemical Co.). After 2 weeks incubation at 28°C in the dark, cultures were filtered through 0.45 micron cellulose acetate filters, and rinsed with Fries mineral salts. Fungal mycelium was suspended in 15 mL of 0.1 MC-FB<sub>1</sub>, pH 5.2 + 1 mM EDTA + 3 µg/mL Pepstatin A + 1.5 µg/mL Leupeptin and disrupted in a Bead Beater™ using 0.5 mm beads and one minute pulses, with ice cooling. Hyphal pieces were collected by



filtering through Spin X (0.22  $\mu$ m), and both mycelial supernatant and original culture filtrates were assayed for fumonisin modification by methods outlined above.

**Preparation of crude culture filtrate.** Agar cultures grown as above were used to inoculate YPD broth cultures (500 ml) in conical flasks at a final concentration of 10<sup>5</sup> cells per ml culture. Cultures were incubated 5 days at 28°C without agitation and mycelia harvested by filtration through 0.45 micron filters under vacuum. The filtrate was discarded and the mycelial mat was washed and resuspended in sterile carbon-free, low mineral salts medium (1 g/liter NH<sub>3</sub>NO<sub>3</sub>; 1 g/liter NaH<sub>2</sub>PO<sub>4</sub>; 0.5 g/liter MgCl<sub>2</sub>; 0.1 g/liter NaCl; 0.13 g/liter CaCl<sub>2</sub>; 0.02 g/liter FeSO<sub>4</sub> · 7H<sub>2</sub>O, pH 4.5) containing 0.5 mg/ml alkaline hydrolyzed crude FB<sub>1</sub>. After 3-5 days at 28°C in the dark with no agitation the cultures were filtered through low protein binding 0.45 micron filters to recover the culture filtrate. Phenylmethyl sulfonyl fluoride (PMSF) was added to a concentration of 2.5 mM and the culture filtrate was concentrated using an Amicon™ YM10 membrane in a stirred cell at room temperature, and resuspended in 50 mM sodium acetate, pH 5.2 containing 10 mM CaCl<sub>2</sub>. The crude culture filtrate (approx. 200-fold concentrated) was stored at -20°C.

To obtain preparative amounts of enzyme-hydrolyzed fumonisin, 10 mg. of FB<sub>1</sub> (Sigma) was dissolved in 20 mL of 50 mM sodium acetate at pH 5.2 + 10 mM CaCl<sub>2</sub>, and 0.25 mL of 200x concentrated crude culture filtrate of 2141.10 was added. The solution was incubated at 37°C for 14 hours, and then cooled to room temperature. The reaction mixture was brought to approx. pH 9.5 by addition of 0.4 mL of 4 N KOH, and the mixture was extracted twice with 10 mL ethyl acetate. The combined organic layers were dried under LN<sub>2</sub> and resuspended in dH<sub>2</sub>O. 2.5 milligrams of organic extracted material were analyzed by Fast Atom Bombardment (FAB) mass spectrometry. The resulting mass spectrum showed a major ion at M/2=406 mass units, indicating the major product of enzymatic hydrolysis was AP<sub>1</sub>, which has a calculated molecular weight of 406.63.

**Additional characterization of fumonisin esterases from *Exophiala spinifera* and Gram-negative bacterium species.** Crude, concentrated culture filtrates (induced for FB<sub>1</sub> esterase activity) from *E. spinifera* isolate 2141.10 and *Xanthomonas* sp. 2412.1 were chromatographed on a Pharmacia® Superdex 75 size exclusion column and eluted with 50 mM sodium phosphate, pH 6.0 containing 0.2 M NaCl. One-mL fractions were collected and assayed for FB<sub>1</sub> esterase activity by methods described above. The retention times for the

2141.10 and 2412.1 FB<sub>1</sub> esterases resulted in estimated molecular weights of 44.5 and 28.7 kilodaltons, respectively.

Similarly, crude concentrated culture filtrates in 1.7 M ammonium sulfate were injected onto a Pharmacia® Phenyl Sepharose FPLC column equilibrated with 1.7 M ammonium sulfate in 50 mM sodium phosphate pH 6.0 (Buffer A). A 30 mL, linear gradient of Buffer A to distilled water was applied, followed by a wash with 0.1% Triton X-100 in 50 mM sodium phosphate, pH 6.0. One-mL fractions were collected and assayed for both FB<sub>1</sub> esterase and for nonspecific esterase (as measured by naphthyl acetate hydrolysis using the method of Dary et al. (1990) "Microplate adaptation of Gomori's assay for quantitative determination," *Journal of Economic Entomology* 83: 2187-2192. Figure 2a and b shows the retention times for the specific (i.e. FB<sub>1</sub>) versus nonspecific (i.e. naphthyl acetate esterase) activities. Both fungal and bacterial FB<sub>1</sub> esterase activity eluted at approx. 0.4 M ammonium sulfate. Naphthyl acetate esterase activity was detected in both fungal and bacterial cultures but this activity did not co-elute with the FB<sub>1</sub> esterase activity. Thus the fungal and bacterial FB<sub>1</sub> esterases are not the same as nonspecific esterases detectable in the culture filtrates of these microbes.

#### Example 2

##### Cloning of genes coding for fumonisin esterase

Microorganisms demonstrating fumonisin-resistance can be used to create a genomic library using standard techniques, well known in the art. Thus, restriction enzymes can be used to render DNA fragments which can in turn be inserted into any number of suitable cloning vectors. Numerous cloning vectors are known to those of skill in the art, and the selection of an appropriate cloning vector is a matter of choice. The cloning vector need only be capable of transforming a host cell incapable of fumonisin degradation. Examples of recombinant DNA vectors for cloning and host cells which they can transform, shown in parentheses, include the bacteriophage lambda (*E. coli*), pBR322 (*E. coli*), pACYC177 (*E. coli*), pKT230 (gram-negative bacteria), pGV1106 (gram-negative bacteria), pLAFRI (gram-negative bacteria), pME290 (non-*E. coli* gram-negative bacteria), pIJ61 (*Streptomyces*), pUC6 (*Streptomyces*), YIp5 (*Saccharomyces*), and YCp19 (*Saccharomyces*). See, generally DNA Cloning, Vols. I and II, *supra*, and Maniatis et al., *supra*. Particularly useful is a cloning vector able to transform *E. coli*.

Once the cloning vector has been inserted into an appropriate host cell, the cells are grown on fumonisin containing media and screened for their ability to degrade fumonisin as previously described. Plasmid DNA inserts from colonies that degrade fumonisin are characterized by subcloning, transposon tagging, and DNA sequence analysis, all well within the skill in the art (see, e.g., Napoli, C., and Staskawicz, B. (1987) *J. Bact.* 169:572-578). Once a coding sequence is determined, recombinant protein molecules able to degrade fumonisin can be produced according to the present invention by constructing an expression cassette and transforming a host cell therewith to provide a cell line or culture capable of expressing the desired protein which is encoded within the expression cassette.

Sequences encoding the fumonisin degradation enzyme can be either prepared directly by synthetic methods based on the determined sequence, or by using the sequence to design oligonucleotide probes to clone the native coding sequence using known techniques. The oligonucleotide probes can be prepared and used to screen a DNA library from an organism able to degrade fumonisin as determined above. The basic strategies for preparing oligonucleotide probes and DNA libraries, as well as their screening by nucleic acid hybridization, are well known to those of ordinary skill in the art. See, e.g., *DNA Cloning*, Vol. I, supra; *Nucleic Acid Hybridization*, supra; *Oligonucleotide Synthesis*, supra; Maniatis et al., supra.

The coding sequence can be comprised entirely of the coding sequence so derived, or such sequences can be fused to other sequences (e.g., leader sequences) so that a fusion protein is encoded. See, e.g., U.S. Patents Nos. 4,431,739; 4,425,437 and 4,338,397, the disclosures of which are hereby incorporated by reference. Once an appropriate coding sequence for the fumonisin-degrading enzyme has been prepared or isolated, it can be cloned into any suitable vector or replicon, known in the art. These vectors are described above, with *E. coli* being the host bacterium particularly preferred.

To complete construction of the expression cassettes, the coding sequence is then operably linked to control sequences such as a promoter, ribosome binding site (for bacterial expression) and, optionally, an operator, so that the DNA sequence encoding the protein is transcribed into messenger RNA in the host cell transformed by the vector containing the expression construction. It is within the skill of the art to operably link the fumonisin-degrading enzyme coding sequence to appropriate control sequences in order to bring about transcription and translation. In general, the coding sequence will be downstream from the

promoter sequence and any expression regulatory regions, such as enhancers or operator sequences. If the coding sequence is linked to a heterologous coding sequence or start codon, then it is important to place the coding sequence in reading frame with the latter. If the intended expression host is procaryotic, then it will also be necessary to include a ribosome binding site among the upstream control sequences. Downstream operably linked control sequences will usually comprise a transcription termination sequence.

The construct can then be inserted into an appropriate expression vector. A number of procaryotic and eucaryotic expression vectors are known in the art. Preferred vectors are procaryotic expression vectors. A particularly preferred host for such vectors is *E. coli*. The fumonisin-degrading enzyme is then produced by growing the host cells transformed by the expression cassette under conditions which cause the expression of the biologically active protein, as indicated by the host cells ability to degrade fumonisin in the medium on which it is grown, as described above. The protein can be isolated from the host cells and purified for further study. If the protein is not secreted, it may be necessary to disrupt the host cells and purify the protein from the cellular lysate. Various purification techniques, such as HPLC, size-exclusion chromatography, electrophoresis, and immunoaffinity chromatography, are known, and the selection of the appropriate purification and recovery method is within the skill of the art.

Similarly, the gene can be inserted into the T-DNA region of a Ti or Ri plasmid derived from *A. tumefaciens* or *A. rhizogenes*, respectively. Thus, expression cassettes can be constructed as above, using these plasmids. Many control sequences are known which when coupled to a heterologous coding sequence and transformed into a host organism show fidelity in gene expression with respect to tissue/organ specificity of the original coding sequence. See, e.g., Benfey, P. N., and Chua, N. H. (1989) *Science* 244: 174-181. Particularly suitable control sequences for use in these plasmids are promoters for constitutive leaf-specific expression of the gene in the various target plants. Other useful control sequences include a promoter and terminator from the nopaline synthase gene (NOS). The NOS promoter and terminator are present in the plasmid pARC2, available from the American Type Culture Collection and designated ATCC 67238. If such a system is used, the virulence (*vir*) gene from either the Ti or Ri plasmid must also be present, either along with the T-DNA portion, or via a binary system where the *vir* gene is present on a separate vector. Such systems, vectors for use therein, and methods of transforming plant cells are described in U.S. Pat. No.

4,658,082; U.S. application Ser. No. 913,914, filed Oct. 1, 1986, as referenced in U.S. Patent 5,262,306, issued November 16, 1993 to Robeson, et al.; and Simpson, R. B., et al. (1986) **Plant Mol. Biol.** 6: 403-415 (also referenced in the '306 patent); all incorporated by reference in their entirety.

5        Once constructed, these plasmids can be placed into *A. rhizogenes* or *A. tumefaciens* and these vectors used to transform cells of plant species which are ordinarily susceptible to *Fusarium* or *Alternaria* infection. For example, non-resistant varieties of tomato (*Lycopersicon esculentum*) are often plagued with such infection and new resistant varieties could be developed to withstand *Alternaria*-induced disease in emerging tomato seedlings, 10 although it should be noted that fusarium wilt in tomato is thought to be caused by *F. oxysporum*, not *F. moniliformis*, and *F. oxysporum* apparently does not produce fumonisin. Several other transgenic plants are also contemplated by the present invention including but not limited to soybean, corn, sorghum, alfalfa, rice, clover, cabbage, banana, coffee, celery, tobacco, cowpea, cotton, melon and pepper. The selection of either *A.* 15 *tumefaciens* or *A. rhizogenes* will depend on the plant being transformed thereby. In general *A. tumefaciens* is the preferred organism for transformation. Most dicotyledons, some gymnosperms, and a few monocotyledons (e.g. certain members of the *Liliales* and *Arales*) are susceptible to infection with *A. tumefaciens*. *A. rhizogenes* also has a wide host range, embracing most dicots and some gymnosperms, which includes members of the *Leguminosae*, 20 *Compositae* and *Chenopodiaceae*. Alternative techniques which have proven to be effective in genetically transforming plants include particle bombardment and electroporation. See e.g. Rhodes, C. A., et al. (1988) **Science** 240, 204-207; Shigemura, K. and Dower, W. J. (1988) **BioTechniques** 6, 742-751; Sanford, J. C., et al. (1987) **Particulate Science & Technology** 5:27-37; and McCabe, D. E. (1988) **BioTechnology** 6:923-926.

25        Once transformed, these cells can be used to regenerate transgenic plants, capable of degrading fumonisin. For example, whole plants can be infected with these vectors by wounding the plant and then introducing the vector into the wound site. Any part of the plant can be wounded, including leaves, stems and roots. Alternatively, plant tissue, in the form of an explant, such as cotyledonary tissue or leaf disks, can be inoculated with these vectors and 30 cultured under conditions which promote plant regeneration. Roots or shoots transformed by inoculation of plant tissue with *A. rhizogenes* or *A. tumefaciens*, containing the gene coding for the fumonisin degradation enzyme, can be used as a source of plant tissue to regenerate

fumonisin-resistant transgenic plants, either via somatic embryogenesis or organogenesis. Examples of such methods for regenerating plant tissue are disclosed in Shahin, E. A. (1985) *Theor. Appl. Genet.* 69:235-240; U.S. Pat. No. 4,658,082; Simpson, R. B., et al. (1986) *Plant Mol. Biol.* 6: 403-415; and U.S. patent applications Ser. Nos. 913,913 and 913,914, both filed Oct. 1, 1986, as referenced in U.S. Patent 5,262,306, issued November 16, 1993 to Robeson, et al.; the entire disclosures therein incorporated herein by reference.

Such transformed cells can also be used to regenerate transgenic plants capable of expressing, in specific tissues or constitutively, depending upon the type of promoter utilized, either the fumonisin degrading enzymes elaborated by *Exophiala spinifera*, ATCC 74269, *Rhinocladia atrovirens*, ATCC 74270, or the bacterium of ATCC 55552, or the AP<sub>1</sub> catabolase elaborated by those strains. Such transgenic plants can be harvested, and the appropriate tissues (seed, for example, if a seed specific promoter were used) can be subjected to large scale protein extraction and purification techniques, and the fumonisin degradation enzymes or AP<sub>1</sub> catabolases can be isolated for use in fumonisin and fumonisin hydrolysis product detoxification processes.

Certain esterases fall into a family that is related by primary sequence and overall structure (Cygler M, Schrag JD, Sussman JL, Harel M, Silman I, Gentry MK, Doctor BP (1993) "Relationship between sequence conservation and 3-Dimensional structure in a large family of esterases, lipases, and related proteins." *Protein Sci* 2: 366-382.). PCR primers were designed based on highly conserved regions of this esterase family and using these primers, a cDNA clone from *Exophiala spinifera* isolate 2141.10 was obtained that showed significant homology to known esterases, and was specifically induced by fumonisin and other inducers. This esterase can be expressed in *E. coli* and its enzyme activity can be measured by means of the TLC assay described above. If no activity is obtained in *E. coli* then expression can be measured in yeast or another eukaryotic system.

Other methods can also be used to clone the gene. Purification of the protein and N-terminal sequencing allow design of specific DNA probes; generation of antibodies from purified protein and screening an expression library; using RNA enrichment methods to obtain cDNAs specific to the induced culture. Once the gene has been confirmed as corresponding to fumonisin esterase, the cDNA clone can easily be ligated into appropriate expression vectors for expression of the enzyme in maize tissue culture cells, transgenic maize, and also in *Fusarium moniliforme* itself, that is useful for studying the mechanisms of pathogenesis

associated with the fungus and its toxin. Transformed or transient-expressing maize tissue culture cells can then be evaluated for resistance to fumonisins relative to control transformed tissue, and in fact fumonisin can be used as a selection agent to isolate transformed cells from tissue culture.

#### 5 Cloning of *Xanthomonas*/*Sphingomonas* Esterase Gene:

The *Xanthomonas* esterase gene was cloned in a lambda ZAP express expression library from Sau3A partially digested bacterial DNA (4-8 kb size selected from ATCC 55552). Pools of lambda lysates were tested for fumonisin esterase assay by TLC using pure fumonisin as a substrate, and positive pools were sub-sampled to enrich for positive clones. Individual  
 10 plaques were resuspended and activity assayed in the lysate. One positive clone was purified, phagemid excised and DNA prepared for sequencing. A 4 kilobase DNA fragment containing fumonisin esterase activity was sequenced and found to contain a 1589 base pair region containing a 529 amino acid open reading frame with high homology to members of the serine carboxylesterase type B superfamily. The open reading frame codes for a hypothetical protein  
 15 (called BEST1) with a putative signal peptide from amino acid 1 to 38, giving a mature protein with a calculated molecular weight of 51,495.63 daltons and a pI of 8.19. This open reading frame showed 52.5% similarity and 34% identity with the amino acid sequence of a rabbit cholinesterase (P37176). Other cholinesterases showed similar homology scores. The BEST1 sequence was also 53.0% similar and 36.4% identical to a *Bacillus subtilis* para-nitrobenzyl esterase (P04058). The open reading frame also showed 54.6% similarity and  
 20 34.9% identity with the *Exophiala spinifera* fumonisin esterase (Esp1). Aside from their overall similarity with other type B carboxylesterases, Esp1 and BEST1 share two short amino acid domains not found in other known esterases of this type:

<u>Protein</u>	<u>Sequence</u>	<u>From</u>	<u>To</u>
ESP1	ATLM	292	295
BEST1	ATLM	286	289
ESP1	TNI	175	177
BEST1	TNI	172	174

25

These domains may be involved in the substrate specificity of these enzymes (Cyglar M., Schrag, J.D., Sussman, J.L. Harel, M., Silman I., Gentry, M.K. Doctor, B.P. (1993)

Relationship between sequence conservation and 3-Dimensional structure in a large family of esterases, lipases, and related proteins. Protein Sci. 2:366-382.).

### Example 3

#### Preparation of AP1-induced and non-induced mycelium.

5        *Exophiala spinifera* isolate 2141.10 was grown in YPD broth for 5 days at 28°C, mycelium was harvested on 05. micron cellulose acetate filters and transferred to fresh medium consisting of Fries mineral salts (Gilchrist DG, Grogan RG (1976) "Production and nature of a host-specific toxin from *Alternaria alternata* f.sp. *lycopersici*." Phytopathology 66: 165-171) amended with hydrolyzed fumonisin B1 (AP1) (0.5 mg/mL) or delta-  
10        aminobutyric acid ( $\delta$ -ABA) (1 mg/mL) as the sole carbon source. Cultures were incubated in the dark for 48 hr at 28°C and culture supernatants removed by filtration through 0.5 micron cellulose acetate. The remaining mycelial mat was washed with sterile Fries mineral salts and then frozen in liquid nitrogen for storage.

### Example 4

#### 15        RNA isolation from *Exophiala spinifera*

The mycelial mats described above (~1 gram) were ground in liquid nitrogen in a mortar and pestle following addition of 10 mL "TRIAGENT" (Molecular Research Center, Inc. Cincinnati, OH) in the presence of 0.2 volume chloroform. The grindate was centrifuged and the resulting supernatant precipitated with isopropanol. The resulting pellet was extracted  
20        with phenol, ethanol precipitated, and stored at -80° C.

The RNA in water (0.4 mL) was enriched for poly-A-containing mRNA using biotin-oligo(dT) and a streptavidin magnetic bead system (Promega) using the manufacturer's instructions. The polyA(+)-enriched RNA was stored at -80° C.

First strand cDNA synthesis from polyA(+)-enriched RNA was carried out using M-  
25        MLV reverse transcriptase (37° C, 1 hr). The reaction mixture was extracted with phenol and chloroform. Aliquots were taken for polymerase chain reaction (PCR) using the degenerate primers identified in SEQUENCE I.D. NOS. 1 through 4. :

ESP5'-OL1    GGGGAATTCGARGAYTGNYTNTAYNTNAAAYRT (SEQUENCE I.D. NO. 1)

30        ESP5'-OL2    GGGGAATTCMCNGTNNTNVTNTGGATNYAYGGNGGGNG (SEQUENCE I.D. NO. 2)



ESP3'-OL1 GGGAAGCTTGGRTYNCCNCCRAANKBNGCDATRTT (SEQUENCE I.D. NO. 3)

ESP3'-OL2 GGGAAGCTTCNCCNGCNSWYTCNCCRAANADNGTNA (SEQUENCE I.D. NO. 4)

- 5 Most bases designated "N" were inosines.

Thermocycler reaction conditions were:

1. 94° C 2 min
2. 94° C 30 sec
3. 45° C 2 min
- 10 4. 72° C 1 min
5. repeat steps 2-4 for 35 X
6. 72° C 5 min

The PCR reaction products were electrophoresed on horizontal agarose gels. Bands that were present only in induced lanes were excised and the DNA was eluted. The recovered

15 DNA was digested with HindIII and EcoRI and ligated into pBluescript SK+.

A recombinant clone from products amplified using ESP5'-OL2 and ESP3'-OL2 (ESP26-1) was recovered and sequenced. The cloned region contains an open reading frame with the partial protein or amino acid sequence

...SFHLYDGASFAANQDVIVVTINYRTNILGFPAAPQLPITQRNLGFLDQRFALDWVQR  
20 NIAAFGGDPRKVT FFGESA... (SEQUENCE I.D. NO. 5)

The above deduced amino acid sequence from DNA fragment ESP26-1 showed significant homology to a family of proteins that includes cholinesterases, acetylcholinesterases, carboxylesterases, and certain lipases (Cygler M, Schrag JD, Sussman JL, Harel M, Silman I, Gentry MK, Doctor BP (1993) "Relationship between  
25 sequence conservation and 3-Dimensional structure in a large family of esterases, lipases, and related proteins." Protein Sci 2: 366-382.)

### Examples 5-6

#### **Comparison of Deduced Amino Acid Sequence to Known Sequences**

In comparison with a sequence published in Arpagaus, M., Chatonnet, A., Masson, P.,  
30 Newton, M., Vaughan, T.A., Bartels, C.F., Nogueira, C.P., La Du, B.N., and Lockridge, O. J. Biol. Chem. 266, 6966-6974 (1991), 43 of the 76 amino acids in ESP26-1 were identical to a dog pancreatic cholinesterase.

In another comparison 32 of 62 amino acids from ESP26-1 were identical to a fungal lipase, as published by Lotti, M., Grandori, R., Fusetti, F., Longhi, S., Brocca, S., Tramontano, A., and Alberghina, L., *Gene* 124, 45-55 (1993).

#### Example 7

##### 5 Northern blot analysis of induced, non-induced *Exophiala spinifera*:

Total RNA extracted from *Exophiala spinifera* cultures as described in the preceding examples was electrophoresed on agarose gels containing formaldehyde, blotted to nitrocellulose, and probed with random-primed <sup>32</sup>P-labelled ESP26-1 cDNA. The probe hybridized to an RNA of approximately 2.0 kilobases in size in the induced lane, but not in the  
10 non-induced lane (see Figure 1).

#### Example 8

##### Isolation of full length cDNA of ESP26-1 from *Exophiala spinifera*.

To obtain 3'-end of the cDNA coding for the putative esterase, a 3'-rapid amplification of cDNA ends protocol (3'-RACE) was employed (Frohman, M.A., Dush, M.K., and Martin, G.R. 1988 "Rapid production of full-length cDNAs from rare transcripts: Amplification using  
15 a single gene-specific oligonucleotide primer." *Proc. Natl. Acad. Sci.* 85: 8998-9002). 5 µg of total RNA isolated from AP1 induced *Exophiala spinifera* mycelia was used as template for reverse transcription reaction. The reverse transcription reaction and subsequent PCR amplification was performed with a 3'-RACE kit (Gibco BRL). The gene-specific primer  
20 (ESP3'-1: GCTAGTTTCGCAGCCAATCA-GGA) (SEQUENCE I.D. NO. 6) was designed based on ESP26-1 sequence.

PCR reaction conditions were:

1. 94° C 4 min
2. 94° C 45 sec
- 25 3. 60° C 25 sec
4. 72° C 3 min
5. repeat steps 2-4 for 40 X
6. 72° C 10 min

A resulting 1.5 kb DNA fragment was blotted to nitrocellulose and hybridized with  
30 cDNA ESP26-1 under highly stringent hybridization and wash conditions (last wash: 0.1 X SSC, 0.5% SDS, 65°C for 30 min.) The DNA fragment was gel-isolated, ligated into a pGEM-T vector (Promega), and transformed into DH5α (Gibco BRL). The resulting plasmid

DNA (p3RC-2) was sequenced using M13 universal primer. Sequence comparison of 3RC-2 and ESP26-1 indicated the ESP26-1 overlapped 100% with the 5' end of 3RC-2 sequence.

To obtain the amino-terminal sequence, a 5'-RACE strategy was employed (Frohman, *et al.*, *supra*). 5 µg of total RNA isolated from API induced *Exophiala spinifera* mycelia was reverse transcribed with SuperScript I RNase H- reverse Transcriptase (Gibco BRL) using an anti-sense primer constructed against ESP26-1 sequence (ESP5'-1:

AAAGGCTGCGATGTTCCGCTGTA) (SEQUENCE I.D. NO. 7). The cDNA was tailed with dATP using terminal transferase (Promega) and used as a template for nested amplification using a second gene-specific anti-sense primer (ESP5'-2:

TCGCTGTGTTATTGGCAGCTGAG. (SEQUENCE I.D. NO. 8). C was a silent mutation of A in order to create a Pvu II restriction site) and an end-blocked polyT primer (BamT17V: CGCGGATCCGTTTTTTTTTTTTTTTTT) (SEQUENCE I.D. NO. 9).

PCR reaction conditions were:

1. 94° C 4 min
2. 94° C 45 sec
3. 40° C 45 sec
4. 60° C 25 sec
5. 72° C 3 min
6. repeat steps 2-5 for 41 X
7. 72° C 10 min

The PCR products were fractionated on a 1.5% agarose gel. The amplified product was gel-isolated, ligated into pGEM-T (Promega), and transformed into DH5 (Gibco BRL). The resulting 5' RACE product was sequenced and shown to overlap as expected with the 3' RACE product and to contain an open reading frame with significant homology to members of the serine esterase/lipase superfamily described by Cygler *et al.* (*supra*). The overlapping sequences obtained by 3' RACE and 5' RACE were combined to yield a cDNA sequence corresponding to the complete open reading frame. The full length, 1937 bp cDNA clone from *Exophiala spinifera* 2141.10 (abbreviated ESP1) contains an open reading frame of 537 amino acids as shown below (SEQUENCE I.D. NO. 10).

MPSRYILSWLLTCFLGLAFGSRGSSAPT VKIDAGMVVGTTTTVPGTTATVSEFLGVVPF  
AASPTRFAPPTRPVPWSTPLQATAYGPACPQQFNYPEELREITMAWFNTPPPSAGED  
CLNLNIYVPGTENTNKAVMVWIYGGALEYGWNSFHLVDGASFAANQDVIVVTINYRT

NILGFPAAPQLPITQRNLGFLDQRFALDWVQRNIAAFGGDPRKVTIFGQSAGGRSVDV  
 LLTSMPHNPPFRAAIMESGVANYNFPKGDLEPWNTTVQALNCTTSIDILSCMRRVDL  
 ATLMNTIEQLGLGFEYTLDNVTVVYRSETARTTGDIARVPVLVGTVANDGLLFLVGEN  
 DTQAYLEEAI PNQPDLYQTLLGAYPIGSPGIGSPQDQLAAIETEVRFCPSAIVAQDSRN  
 5 RGIPSWRYYYNATFENLELFPGSEVYHSSEVGMVFGTYPVASATALEAQTSKYMQGA  
 WAAFAKNPMNGPGWKQVPNVAALGSPGKAIQVDVSPATIDQRCALYTHYYTELGTIA  
 PRTF

This open reading frame (ORF) shows some homology to members of the serine  
 esterase/lipase superfamily described by Cygler et al. (supra). The most extensive homology is  
 10 35.9% identity in 320 amino acid overlap with butyrylcholinesterase from *Oryctolagus*  
*cuniculus* (rabbit).

The deduced Esp1 protein contains a putative signal peptide which is probably cleaved  
 at position 26/27 yielding a mature protein with a calculated MW of 54953.781 and  
 calculated pI of 4.5. These calculated values are consistent with the estimated MR and pI of  
 15 the fumonisin esterase activity described above.

A comparison of the Esp1 open reading frame consensus regions in the esterase  
 superfamily (Cygler et al., supra) reveals numerous conserved features indicating Esp1 may  
 code for a serine esterase. The Esp protein has a potential serine active site consensus at 223-  
 228; a putative aspartate active site consensus at 335-341 that is typical of cholesterol  
 20 esterases and *Drosophila* 6 and P proteins [the majority of members of this superfamily,  
 including fungal lipases and carboxylesterases have glutamate at the active site instead of  
 aspartate]; and a putative histidine active site that is different from any members of the family,  
 containing additional amino acids between the G and H. The putative Esp mature protein has  
 a total of 6 cysteines, for 3 possible disulfide bridges, consistent with at least a subset of the  
 25 esterases in the superfamily described by Cygler et al., supra

Thus the Esp ORF has most of the hallmarks of a bona fide member of the  
 lipase/esterase superfamily, including a putative active site triad and other conserved amino  
 acids. The regions of conservation are not consistent with any one substrate subgroup (i.e.  
 lipase, cholinesterase, carboxylesterase, or cholesterol esterase), but seem to be contain some  
 30 features of several of these, and Esp appears to be unique among known esterases in its  
 putative active site His consensus sequence.

**Example 9****Effect of FB<sub>1</sub> and AP<sub>1</sub> on maize coleoptiles**

Maize coleoptiles from 4 day dark-grown germinated maize seeds were excised above the growing point and placed in 96-well microtiter plates in the presence of 60 microliters of sterile distilled water containing FB<sub>1</sub> or AP<sub>1</sub> at approximately equimolar concentrations of 1.5, .5, .15, .05, .015, .005, .0015, or .0005 millimolar, along with water controls. After 2 days in the dark at 28° C the coleoptiles were placed in the light and incubated another 3 days. Injury or lack thereof was evaluated as follows:

	0	.0005	.0015	.005	.015	.05	.15	.5	1.5	mM
FB <sub>1</sub>	-	-	-	-	+/-	+	+	+	+	
AP <sub>1</sub>	-	-	-	-	-	-	-	-	+	

+ = brown necrotic discoloration of coleoptile

- = no symptoms (same as water control)

The results (see table above) indicate there is at least a 30-fold difference in toxicity between FB<sub>1</sub> and AP<sub>1</sub> to maize coleoptiles of this genotype. This is in general agreement with other studies where the toxicity of the two compounds was compared for plant tissues: In *Lemna* tissues, AP<sub>1</sub> was approx. 40-fold less toxic (Vesonder RF, Peterson RE, Labeda D, Abbas HK (1992) "Comparative phytotoxicity of the fumonisins, AAL-Toxin and yeast sphingolipids in *Lemna minor* L (Duckweed)." *Arch Environ Contam Toxicol* 23: 464-467.). Studies with both AAL toxin and FB<sub>1</sub> in tomato also indicate the hydrolyzed version of the molecule is much less toxic (Gilchrist DG, Ward B, Moussato V, Mirocha CJ (1992) "Genetic and Physiological Response to Fumonisin and AAL-Toxin by Intact Tissue of a Higher Plant." *Mycopathologia* 117: 57-64.). In a recent report Lamprecht *et al.* also observed an approximate 100-fold reduction in toxicity to tomato by AP<sub>1</sub> versus FB<sub>1</sub> (Lamprecht S, Marasas W, Alberts J, Cawood M, Gelderblom W, Shephard G, Thiel P, Calitz J (1994) Phytotoxicity of fumonisins and TA-toxin to corn and tomato. *Phytopathology* 84: 383391.)

**Example 10****Effect of FB<sub>1</sub> and AP<sub>1</sub> on maize tissue cultured cells (Black Mexican Sweet, BMS)**

FB<sub>1</sub> or AP<sub>1</sub> at various concentrations was added to suspensions of BMS cells growing in liquid culture medium in 96-well polystyrene plates. After 1 week the cell density in wells was observed under low power magnification and growth of toxin-treated wells was compared to control wells that received water. Growth of BMS cells was significantly inhibited at 0.4

micromolar FB<sub>1</sub>, but no inhibition was observed until 40 micromolar AP<sub>1</sub>. This represents an approximate 100-fold difference in toxicity to maize tissue cultured cells. Similarly Van Asch et al. (Vanasch MAJ, Rijkenberg FHJ, Coutinho TA (1992) "Phytotoxicity of fumonisin b<sub>1</sub>, moniliformin, and t-2 toxin to corn callus cultures." *Phytopathology* 82: 1330-1332)

5 observed significant inhibition of maize callus grown on solid medium at 1.4 micromolar. AP<sub>1</sub> was not tested in that study, however.

### Example 11

#### **AP<sub>1</sub> Catabolase Activity**

A cell-free extract that contains the catabolase activity was obtained by subjecting  
10 substrate-induced *Exophiala spinifera* cells to disruption using a bead beater in sodium acetate buffer, pH 5.2, and recovering the cell-free supernatant by centrifugation and .45 micron filtration. Catabolic activity is assayed by incubating extracts with AP<sub>1</sub> (hydrolyzed fumonisin B<sub>1</sub> backbone) or 14C-labelled with the extract and evaluating by TLC on C18 silica. The product AP<sub>1</sub>-N<sub>1</sub> has a lower R<sub>f</sub> than AP<sub>1</sub> and is detected either by radiolabel scan or by H<sub>2</sub>SO<sub>4</sub>  
15 spray/charring of the TLC plate. AP<sub>1</sub>-N<sub>1</sub> does not react with the amine reagent, fluorescamine, that is routinely used to detect AP<sub>1</sub> on TLC plates, suggesting that the amine group is missing or chemically modified. Activity is greater at 37°C than at room temperature, but following 30 min. at 65°C or 100°C (no AP<sub>1</sub> catabolic activity remained). Activity is maximal at pH to 9. At pH 9, complete conversion to AP<sub>1</sub>-N<sub>1</sub> occurred in 30 minutes.  
20 Activity is retained by 30,000 dalton molecular weight cutoff membrane, but only partially retained by 100,000 dalton molecular weight cutoff membrane. Other amine-containing substrates were tested for modification by the crude extract. fumonisin (with tricarboxylic acids attached) is not modified by the extract, indicating that hydrolysis must occur first for the catabolase to be active. Other long-chain bases (sphingosine, sphinganine, phytosphingosine)  
25 are apparently not modified by the crude catabolase, suggesting the enzyme(s) is specific for the fumonisin backbone. Preparative amounts of the product, tentatively named AP<sub>1</sub>-N<sub>1</sub>, have also been purified and analyzed by C13 nmr. The results indicate that AP<sub>1</sub>-N<sub>1</sub> lack an amino nitrogen, and that it probably contains a keto function. This may point to either an amine oxidase or an ammonia lyase enzyme. The product of either enzyme would not be expected to  
30 display any significant toxicity (although this has not been tested).

### Example 12

#### **Demonstration of Functional Esterase Activity**

Demonstration of esterase activity was accomplished by expressing the *E. spinifera* cDNA in two heterologous systems (an insect cell/baculovirus expression system, and transgenic maize obtained by microprojectile bombardment) and subsequently detecting fumonisin esterase activity in transformed tissue. Forty-four maize calli (Hi type II) were bombarded with the esterase gene fused to the ubiquitin promoter plus a selectable marker (PAT) plasmid. Using thin layer chromatographic analysis, 38 lines were positive for fumonisin hydrolysis based upon the presence of a free tricarboxylic acid spot in the TLC plates. Four negative control calli had no such spot. Similar results were obtained with insect cells infected with a baculovirus expression vector containing the esterase clone.

Esterase activity was also detected in leaf tissue of regenerated T0 maize plants. Ten leaf punches (4mm dia) were taken from the 6th leaf of regenerated plants of roughly the same age (7 leaf stage) representing four classes of transformants: selectable marker (Bar) only, low esterase (+), medium esterase (++) and high esterase (+++) expressors (based on callus data). Four plants, representing different events in each class, were sampled. The punches were suspended in 200 microliters of 50 mM sodium acetate buffer, pH 5.2, containing leupeptin and pepstatin to inhibit proteinases, and homogenized by rapid agitation with a 5/32" steel bead, twice for 30 sec. The homogenates were spun at 4000 rpm, 10 min. at 4C and 150 microliters supernatant recovered. Samples were assayed for protein concentration (Bradford assay) and adjusted to the same protein concentration and then assayed for fumonisin esterase activity using <sup>14</sup>C-FB1 as a substrate. After 30 min. or 4 hrs. reactions were spotted on TLC plates (C18) and developed with MeOH:4%KCl (3:2). Plates were scanned with a radiometric scanner (AMBIS) and scored for fumonisin esterase activity on a product), ++ (up to 50% conversion to product) and +++ (between 90-100% conversion to product).

Results were as follows:

SAMPLE	CALLUS SCORE (30 MIN)	30 MIN. LEAF SCORE	4 HR. LEAF SCORE
A1	+	-	-
B1 Control	-	-	-
C1	++	-	-
D1	+	-	+
E1 Control	-	-	-
F1	+++	+	++
G1	+++	+	+++
H1	++	+	++
A2	+	-	-
B2 Control	-	-	-
C2 Control	-	-	-
D2	+++	+++	+++
E2	++	+	++
F2	-	-	-
G2	+++	+	+++
H2	+++	+	+++

In summary, 8 or 12 callus expressors were positive for leaf expression. All negative  
 5 controls plus four callus expressors were negative. Of the four "+++" callus expressors, only  
 one (D2) had the same high level (30 min. assay), but all were positive.

### Example 13

#### **Detoxification of Harvested Grain**

10 The present invention also relates to a method of detoxifying a fumonisin or a  
 structurally related mycotoxin with an enzyme having the structure of the fumonisin  
 degradative enzymes or the AP<sub>1</sub> catabolase elaborated by *Exophiala spinifera*, ATCC 74269,  
*Rhinocladiella atravirens*, ATCC 74210, or the bacterium of ATCC 55552 during the  
 processing of grain for animal or human food consumption. Since the atmospheric  
 15 ammoniation of corn has proven to be an ineffective method of detoxification (see B. Fitch  
 Haumann, "Eradicating Mycotoxin in Food and Feeds," INFORM 6:248-257 (1995)), such a  
 methodology is particularly critical where transgenic detoxification is not applicable.



In this embodiment, the fumonisin degradative enzyme and/or the AP<sub>1</sub> catabolase elaborated by *Expohilia spinifera*, ATCC 74269, *Rhinocladia atra*, ATCC 74210, or the bacterium of ATCC 55552, are presented to grain during the processing procedure, at the appropriate stages of the procedure and in amounts effective for detoxification of fumonisins and structurally related mycotoxins. Detoxification by this method can occur not only during the grain processing, but also any time prior to feeding of the grain to an animal or incorporation of the grain into a human food product.

The enzymes can be introduced during processing in appropriate manners, for example as a wash or spray, or in dried or lyophilized form or powdered form, depending upon the nature of the milling process and/or the stage of processing at which the enzymatic treatment is carried out. See generally, Hoseney, R.C., Principles of Cereal Science and Technology, American Assn. of Cereal Chemists, Inc., 1990 (especially Chapters 5, 6 and 7); Jones, J.M., Food Safety, Eagan Press, St. Paul, MN, 1992 (especially Chapters 7 and 9); and Jelen, P., Introduction to Food Processing, Reston Publ. Co., Reston, VA, 1985. Processed grain to be used for animal feed can be treated with an effective amount of the enzymes in the form of an inoculant or probiotic additive, for example, or in any form recognized by those skilled in the art for use in animal feed. The enzymes of the present invention are expected to be particularly useful in detoxification during processing and/or in animal feed prior to its use, since the enzymes display relatively broad ranges of pH activity. The esterase from *Exophilia spinifera*, ATCC 74269, showed a range of activity from about pH 3 to about pH 6, and the esterase from the bacterium of ATCC 55552 showed a range of activity from about pH 6 to about pH 9.

#### Example 14

##### **Genetic Engineering of Ruminant Microorganisms**

Ruminant microorganisms can be genetically engineered to contain and express either the fumonisin degrading enzymes or the AP<sub>1</sub> catabolase elaborated by *Exophilia spinifera*, ATCC 74269, *Rhinocladia atra*, ATCC 74270, or the bacterium of ATCC 55552, or a combination of the enzymes. The genetic engineering of microorganisms is now an art recognized technique, and ruminant microorganisms so engineered can be added to feed in any art recognized manner, for example as a probiotic or inoculant. In addition, microorganisms capable of functioning as bioreactors can be engineered so as to be capable of mass producing either the fumonisin degrading enzymes or the AP<sub>1</sub> catabolase elaborated by *Exophilia*

*spinfera*, ATCC 74269, *Rhinocycladiella atravirens*, ATCC 74270, or the bacterium of ATCC 55552.

## SEQUENCE LISTING

## (1)- GENERAL INFORMATION:

(i) APPLICANT: Duvick, Jon  
Rood, Tracy A.

5 (ii) TITLE OF INVENTION: FUMONISIN DETOXIFICATION  
COMPOSITIONS AND METHODS

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Pioneer Hi-Bred International

10 (B) STREET: 700 Capital Square, 400 Locust Street

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(E) COUNTRY: USA

(F) ZIP: 50309

15 (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: MS-DOS

(D) SOFTWARE: Microsoft Windows 3.1 - Notepad

20 (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

(B) FILING DATE:

(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

25 (A) NAME: Roth, Michael J.

(B) REGISTRATION NUMBER: 29,342

(C) REFERENCE/DOCKET NUMBER: 0272 US

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: 515-248-4895

30 (B) TELEFAX: 515-248-4934

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 32 bases

(B) TYPE: nucleotide

35 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: probe  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  
5 GGGGAATTCG ARGAYTGNYT NTAYNTNAAAY RT 32  
(2) INFORMATION FOR SEQ ID NO:2:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 37 bases  
    (B) TYPE: nucleotide  
10      (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: probe  
(iii) HYPOTHETICAL: NO  
(iv) ANTI-SENSE: NO  
15      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:  
GGGGAATTCM CNGTNNTNVT NTGGATNYAY GGNGGNG 37  
(2) INFORMATION FOR SEQ ID NO:3:  
(i) SEQUENCE CHARACTERISTICS:  
    (A) LENGTH: 35 bases  
20      (B) TYPE: nucleotide  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: probe  
(iii) HYPOTHETICAL: NO  
25      (iv) ANTI-SENSE: NO  
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:  
GGGAAGCTTG GRTYNCCNCC RAANKBNGCD ATRTT 35  
(2) INFORMATION FOR SEQ ID NO:4:  
(i) SEQUENCE CHARACTERISTICS:  
30      (A) LENGTH: 36 bases  
    (B) TYPE: nucleotide  
    (C) STRANDEDNESS: single  
    (D) TOPOLOGY: linear  
(ii) MOLECULE TYPE: probe  
35      (iii) HYPOTHETICAL: NO  
    (iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGAAGCTTC NCCNGCNSWY TCNCCRAANA DNGTNA

36

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 76 amino acid  
(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein (partial)

10 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

	Ser	Phe	His	Leu	Tyr	Asp	Gly	Ala	Ser	Phe	Ala	Ala	Asn	Gln	Asp
15	Val	Ile	Val	Val	Thr	Ile	Asn	Tyr	Arg	Thr	Asn	Ile	Leu	Gly	Phe
	Pro	Ala	Ala	Pro	Gln	Leu	Pro	Ile	Thr	Gln	Arg	Asn	Leu	Gly	Phe
	Leu	Asp	Gln	Arg	Phe	Ala	Leu	Asp	Trp	Val	Gln	Arg	Asn	Ile	Ala
20															
	Ala	Phe	Gly	Gly	Asp	Pro	Arg	Lys	Val	Thr	Phe	Phe	Gly	Glu	Ser

Ala

(2) INFORMATION FOR SEQ ID NO:6:

25 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 bases  
(B) TYPE: nucleotide  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: probe

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCTAGTTTCG CAGCCAATCA GGA

23

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bases

(B) TYPE: nucleotide

5 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: probe

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAAGGCTGCG ATGTTCCGCT GTA

23

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 bases

15 (B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: probe

(iii) HYPOTHETICAL: NO

20 (iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TCGCTGTGTT ATTGGCAGCT GAG

23

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 28 bases

(B) TYPE: nucleotide

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: probe

30 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGCGGATCCG TTTTTTTTTT TTTTTTV

28

## (2) INFORMATION FOR SEQ ID NO:10:

35 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 527 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

5 (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

	Met	Pro	Ser	Arg	Tyr	Ile	Leu	Ser	Trp	Leu	Leu	Thr	Cys	Phe	Leu	
						5				10					15	
10	Gly	Ile	Ala	Phe	Gly	Ser	Arg	Cys	Gly	Ser	Ser	Ala	Pro	Thr	Val	
						20				25					30	
	Lys	Ile	Asp	Ala	Gly	Met	Val	Val	Gly	Thr	Thr	Thr	Thr	Val	Pro	
						35				40					45	
	Gly	Thr	Thr	Ala	Thr	Val	Ser	Glu	Phe	Leu	Gly	Val	Pro	Phe	Ala	
15						50				55					60	
	Ala	Ser	Pro	Thr	Arg	Phe	Ala	Pro	Pro	Thr	Arg	Pro	Val	Pro	Trp	
						65				70					75	
	Ser	Thr	Pro	Leu	Gln	Ala	Thr	Ala	Tyr	Gly	Pro	Ala	Cys	Pro	Gln	
						80				85					90	
20	Gln	Phe	Asn	Tyr	Pro	Glu	Glu	Leu	Arg	Glu	Ile	Thr	Met	Ala	Trp	
						95				100					105	
	Phe	Asn	Thr	Pro	Pro	Pro	Ser	Ala	Gly	Glu	Ser	Glu	Asp	Cys	Leu	
						110				115					120	
	Asn	Leu	Asn	Ile	Tyr	Val	Pro	Gly	Thr	Glu	Asn	Thr	Asn	Lys	Ala	
25						125				130					135	
	Val	Met	Val	Trp	Ile	Tyr	Gly	Gly	Ala	Leu	Glu	Tyr	Gly	Trp	Asn	
						140				145					150	
	Ser	Phe	His	Leu	Tyr	Asp	Gly	Ala	Ser	Phe	Ala	Ala	Asn	Gln	Asp	
						155				160					165	
30	Val	Ile	Val	Val	Thr	Ile	Asn	Tyr	Arg	Thr	Asn	Ile	Leu	Gly	Phe	
						170				175					180	
	Pro	Ala	Ala	Pro	Gln	Leu	Pro	Ile	Thr	Gln	Arg	Asn	Leu	Gly	Phe	
						185				190					195	
	Leu	Asp	Gln	Arg	Phe	Ala	Leu	Asp	Trp	Val	Gln	Arg	Asn	Ile	Ala	
35						200				205					210	
	Ala	Phe	Gly	Gly	Asp	Pro	Arg	Lys	Val	Thr	Ile	Phe	Gly	Gln	Ser	

		215		220		225
	Ala Gly Gly Arg Ser Val Asp Val Leu Leu Thr Ser Met Pro His					
		230		235		240
	Asn Pro Pro Phe Arg Ala Ala Ile Met Glu Ser Gly Val Ala Asn					
5		245		250		255
	Tyr Asn Phe Pro Lys Gly Asp Leu Ser Glu Pro Trp Asn Thr Thr					
		260		265		270
	Val Gln Ala Leu Asn Cys Thr Thr Ser Ile Asp Ile Leu Ser Cys					
		275		280		285
10	Met Arg Arg Val Asp Leu Ala Thr Leu Met Asn Thr Ile Glu Gln					
		290		295		300
	Leu Gly Leu Gly Phe Glu Tyr Thr Leu Asp Asn Val Thr Val Val					
		305		310		315
	Tyr Arg Ser Glu Thr Ala Arg Thr Thr Gly Asp Ile Ala Arg Val					
15		320		325		330
	Pro Val Leu Val Gly Thr Val Ala Asn Asp Gly Leu Leu Phe Val					
		335		340		345
	Leu Gly Glu Asn Asp Thr Gln Ala Tyr Leu Glu Glu Ala Ile Pro					
		350		355		360
20	Asn Gln Pro Asp Leu Tyr Gln Thr Leu Leu Gly Ala Tyr Pro Ile					
		365		370		375
	Gly Ser Pro Gly Ile Gly Ser Pro Gln Asp Gln Ile Ala Ala Ile					
		380		385		390
	Glu Thr Glu Val Arg Phe Gln Cys Pro Ser Ala Ile Val Ala Gln					
25		395		400		405
	Asp Ser Arg Asn Arg Gly Ile Pro Ser Trp Arg Tyr Tyr Tyr Asn					
		410		415		420
	Ala Thr Phe Glu Asn Leu Glu Leu Phe Pro Gly Ser Glu Val Tyr					
		425		430		435
30	His Ser Ser Glu Val Gly Met Val Phe Gly Thr Tyr Pro Val Ala					
		440		445		450
	Ser Ala Thr Ala Leu Glu Ala Gln Thr Ser Lys Tyr Met Gln Gly					
		455		460		465
	Ala Trp Ala Ala Phe Ala Lys Asn Pro Met Asn Gly Pro Gly Trp					
35		470		475		480



Lys	Gln	Val	Pro	Asn	Val	Ala	Ala	Leu	Gly	Ser	Pro	Gly	Lys	Ala	
				485					490					495	
Ile	Gln	Val	Asp	Val	Ser	Pro	Ala	Thr	Ile	Asp	Gln	Arg	Cys	Ala	
				500					505					510	
5	Leu	Tyr	Thr	His	Tyr	Tyr	Thr	Glu	Leu	Gly	Thr	Ile	Ala	Pro	Arg
				515						520					525
	Thr	Phe													

**WHAT IS CLAIMED IS:**

1. A method of detoxifying a fumonisin or a structurally related mycotoxin, present in harvested grain, the method comprising reacting the fumonisin with an enzyme having the structure of the fumonisin degradative enzyme elaborated by *Exophiala spinifera*,  
5 ATCC 74269, *Rhinoctadiella atrovirens*, ATCC 74270, or the bacterium of ATCC 55552.
2. The method of Claim 1 wherein the detoxification reaction occurs during storage of the harvested grain.
3. The method of Claim 1 wherein the detoxification reaction occurs during processing of the harvested grain.
- 10 4. The method of Claim 1 wherein the detoxification reaction occurs in processed grain which is to be used as animal feed.
5. A method of detoxifying a fumonisin, a structurally related mycotoxin, a fumonisin hydrolysis product, or a hydrolysis product of a structurally related mycotoxin, present in harvested grain, the method comprising reacting the hydrolysis product with an AP<sub>1</sub> catabolase elaborated by *Exophiala spinifera*, ATCC 74269, *Rhinoctadiella atrovirens*, ATCC  
15 74270, or the bacterium of ATCC 55552.
6. The method of Claim 5 wherein the detoxification reaction occurs during storage of the harvested grain.
7. The method of Claim 5) wherein the detoxification reaction occurs during  
20 processing of the harvested grain.
8. The method of Claim 5 wherein the detoxification reaction occurs in processed grain which is to be used as animal feed.
9. A method for detoxifying, in harvested grain, a fumonisin or a structurally related mycotoxin and for detoxifying a fumonisin hydrolysis product or a hydrolysis product  
25 of a structurally related mycotoxin, the method comprising reacting the fumonisin with an enzyme having the structure of the fumonisin degradative enzyme elaborated by *Exophiala spinifera*, ATCC 74269, *Rhinoctadiella atrovirens*, ATCC 74270, or the bacterium of ATCC 55552, and reacting the hydrolysis product with an AP<sub>1</sub> catabolase elaborated by *Exophiala spinifera*, ATCC 74269, *Rhinoctadiella atrovirens*, ATCC 74270, or the bacterium of ATCC  
30 55552.
10. The method of Claim 9 wherein the detoxification reaction occurs during storage of the harvested grain.

11. The method of Claim 9 wherein the detoxification reaction occurs during processing of the harvested grain.

12. The method of Claim 9 wherein the detoxification reaction occurs in processed grain which is to be used as animal feed.

5 13. A transgenic plant capable of expressing the fumonisin degradative enzyme elaborated by *Exophiala spinifera*, ATCC 74269, *Rhinocladiella atrovirens*, ATCC 74270, or the bacterium of ATCC 55552.

10 14. A transgenic plant capable of expressing the AP<sub>1</sub> catabolase elaborated by *Exophiala spinifera*, ATCC 74269, *Rhinocladiella atrovirens*, ATCC 74270, or the bacterium of ATCC 55552.

15 15. A method of producing the fumonisin degradative enzyme elaborated by *Exophiala spinifera*, ATCC 74269, *Rhinocladiella atrovirens*, ATCC 74270, or the bacterium of ATCC 55552, the method comprising producing a transgenic plant which expresses one or more of said fumonisin degradative enzymes and isolating and purifying the enzymes from the plant tissues expressing the enzymes.

20 16. A method of producing the AP<sub>1</sub> catabolase elaborated by *Exophiala spinifera*, ATCC 74269, *Rhinocladiella atrovirens*, ATCC 74270, or the bacterium of ATCC 55552, the method comprising producing a transgenic plant which expresses one or more of said AP<sub>1</sub> catabolases and isolating and purifying the enzymes from the plant tissues expressing the enzymes.

25 17. A genetically engineered ruminal microorganism comprising an expression vector capable of expressing proteins in microorganisms, said vector comprising a nucleotide sequence encoding the fumonisin degradative enzyme elaborated by *Exophiala spinifera*, ATCC 74269, *Rhinocladiella atrovirens*, ATCC 74270, or the bacterium of ATCC 55552.

18. A probiotic composition comprising the genetically engineered microorganisms of Claim 17.

19. A feed inoculant composition comprising the genetically engineered microorganism of Claim 17.

30 20. A genetically engineered microorganism comprising an expression vector capable of expressing proteins in microorganisms, said vector comprising a nucleotide sequence encoding the fumonisin degradative enzyme elaborated by *Exophiala spinifera*, ATCC 74269, *Rhinocladiella atrovirens*, ATCC 74270, or the bacterium of ATCC 55552.

21. A genetically engineered ruminal microorganism comprising an expression vector capable of expressing proteins in microorganisms, said vector comprising a nucleotide sequence encoding the AP<sub>I</sub> catabolase elaborated by *Exophilia spinifera*, ATCC 74269, *Rhinocycladiella atravirens*, ATCC 74270, or the bacterium of ATCC 55552.
- 5 22. A probiotic composition comprising the genetically engineered microorganisms of Claim 21.
23. A feed inoculant composition comprising the genetically engineered microorganism of Claim 21.
- 10 24. A genetically engineered microorganism comprising an expression vector capable of expressing proteins in microorganisms, said vector comprising a nucleotide sequence encoding the AP<sub>I</sub> elaborated by *Exophilia spinifera*, ATCC 74269, *Rhinocycladiella atravirens*, ATCC 74270, or the bacterium of ATCC 55552.

## FIGURE 1

ACTAGTGGAT	CATTGCATTG	GCTGGCGGAC	TGGCGCGCCG	ATAGTCGTTG	1
CGATGGTCGC	GAGAATAAGC	GTGCGAAGTG	GGAGGATGTG	AAGATGGGGG	51
CCAGGAGTAT	GTGTGCGGGA	CGGTTCCGAC	GCTTCTGCAT	TGGCTTGGCT	101
TCATCGGTTG	CCGTGACTCT	AGGGGGAGCC	TCCGCCGCCG	GCGCGGCAAC	151
CGCGACGGAT	TTTCCGGTCC	GCAGGACCGA	TCTGGGCCAG	GTTTCAGGGAC	201
TGGCCGGGGA	CGTGATGAGC	TTTCGCGGAA	TACCCTATGC	AGCGCCGCCG	251
GTGGGCGGGC	TGCGTTGGAA	GCCGCCCCAA	CACGCCCGGC	CCTGGGCGGG	301
GGTTCGCCCC	GCCACCCAAT	TTGGCTCCGA	CTGCTTCGGC	GCGGCCTATC	351
TTGCAAAGG	CAGCCTCGCC	CCCGGCGTGA	GCGAGGACTG	TCTTTACCTC	401
AACGTATGGG	CGCCGTCAGG	CGCTAAACCC	GGCCAGTACC	CCGTCATGGT	451
CTGGGTCTAC	GGCGGCGGCT	TCGCCGGCGG	CACGGCCGCC	ATGCCCTACT	501
ACGACGGCGA	GGCGCTTGCG	CGACAGGGCG	TCGTCGTGGT	GACGTTTAAC	551
TATCGGACGA	ACATCCTGGG	CTTTTTCGCC	CATCCTGGTC	TCTCGCGCGA	601
GAGCCCCACC	GGAACCTCGG	GCAACTACGG	CCTACTCGAC	ATTCTCGCCG	651
CTCTTCGGTG	GGTGCAGAGC	AACGCCCGCG	CCTTCGGAGG	GGACCCCGGC	701
CGAGTGACGG	TCTTTGGTGA	ATCGGCCGGA	GCGAGCGCGA	TCGGACTTCT	751
GCTCACCTCG	CCGCTGAGCA	AGGGTCTCTT	CCGTGGCGCT	ATCCTCGAAA	801
GTCCAGGGCT	GACGCGACCG	CTCGCGACGC	TCGCCGACAG	CGCCGCCTCG	851
GGCGAGCGCC	TCGACGCCGA	TCTTTCGCGA	CTGCGCTCGA	CCGACCCAGC	901
CACCCTGATG	GCGCGCGCCG	ACGCGGCCCG	CCCGGCATCG	CGGGACCTGC	951
GCAGGCCGCG	TCCGACCGGA	CCGATCGTCG	ATGGCCATGT	GCTGCCGCGAG	1001
ACCGACAGCG	CGGCGATCGC	GGCGGGGCAG	CTGGCGCCGG	TTCGGGTCCT	1051
GATCGGAACC	AATGCCGACG	AAGGCCGCGC	CTTCCTCGGG	CGCGCGCCGA	1101
TGGAGACGCC	AGCGGACTAC	CAAGCCTATC	TGGAGGCGCA	GTTTGGCGAC	1151
CAAGCCGCCG	CCGTGGCGGC	GTGCTATCCC	CTCGACGGCC	GGGCCACGCC	1201
CAAGGAAATG	GTCGCGCGCA	TCTTCGGCGA	CAATCAGTTC	AATCGGGGGG	1251
TCTCGGCCTT	CTCGGAAGCG	CTTGTCGCC	AGGGCGCGCC	CGTGTGGCGT	1301
TATCAGTTCA	ACGGTAATAC	CGAGGGTGGA	AGAGCGCCGG	CTACCCACGG	1351
AGCCGAAATT	CCCTACGTTT	TCGGGGTGTT	CAAGCTCGAC	GAGTTGGGTC	1401
TGTTGATTG	GCCGCCCCGAG	GGGCCACGC	CCGCCGACCG	TGCGCTGGGC	1451
CAACTGATGT	CCTCCGCCTG	GGTCCGGTTC	GCCAAGAATG	GCGACCCCGC	1501
CGGGGACGCC	CTTACCTGGC	CTGCCTATTC	TACGGGCAAG	TCGACCATGA	1551
CATTCGGTCC	CGAGGGCCGC	GCGGCGGTGG	TGTCGCCCGG	ACCTTCCATC	1601
CCCCCTTGCG	CGGATGGCGC	CAAGGCGGGG	TGACGCCGTC	GACGATGGCG	1651
TGACGACGGT	CGAGGCGATG	TTCTCGATCT	GGAGTCCGCG	CCGCCTCGAT	1701
TTGCGTCGTC	TCCGGCGCTC	AGACGAACGC	CCCAGTTCCA	TCCACACAGT	1751

## FIGURE 2

MGARSMCAGR	FGRFCIGLAS	SVAVTLGGAS	AAGAATATDF	PVRRTDLGQV	1
QGLAGDVMSF	RGIPYAAPPV	GGLRWKPPQH	ARPWAGVRPA	TQFGSDCFGA	51
AYLRKGSLAP	GVSEDCLYLN	VWAPSGAKPG	QYPVMVWVYG	GGFAGGTAAM	101
PYYDGEALAR	QGVVVVTFNY	RTNILGFFAH	PGLSRESPTG	TSGNYGLLDI	151
LAALRWVQSN	ARAFGGDPGR	VTVFGESAGA	SAIGLLLTSP	LSKGLFRGAI	201
LESPGLTRPL	ATLADSAASG	ERLDADLSRL	RSTD PATLMA	RADAARPASR	251
DLRRPRPTGP	IVDGHVLPQT	DSAAIAAGQL	APVRVLIGTN	ADEGRAFLGR	301
APMETPADYQ	AYLEAQFGDQ	AAAVAACYPL	DGRATPKEMV	ARIFGDNQFN	351
RGVSAFSEAL	VRQGAPVWRY	QFNGNTEGGR	APATHGAEIP	YVFGVFKLDE	401
LGLFDWPPEG	PTPADRALGQ	LMSSAWVRFA	KNGDPAGDAL	TWPAYSTGKS	451
TMTFGPEGRA	AVVSPGPSIP	PCADGAKAG*			501

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WORLD INTELLECTUAL PROPERTY  
International Bi

INTERNATIONAL APPLICATION PUBLISHED UNDER

WO 9606175A3

(51) International Patent Classification 6:

C12N 15/55, A01N 63/02, C12N 15/82,  
A01H 5/00, C12N 1/21

A3

(11) International Publication Number:

WO 96/06175

(43) International Publication Date:

29 February 1996 (29.02.96)

(21) International Application Number: PCT/US95/10284

(22) International Filing Date: 11 August 1995 (11.08.95)

(30) Priority Data:

08/289,595

12 August 1994 (12.08.94)

US

08/484,815

7 June 1995 (07.06.95)

US

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Locust Street, Des Moines, IA 50309 (US).(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH,  
CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE,  
KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN,  
MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK,  
TJ, TM, TT, UA, UG, UZ, VN, European patent (AT, BE,  
CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT,  
SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML,  
MR, NE, SN, TD, TG). ARIPO patent (KE, MW, SD, SZ,  
UG).

Published

With international search report.

Before the expiration of the time limit for amending the  
claims and to be republished in the event of the receipt of  
amendments.

(88) Date of publication of the international search report:

14 March 1996 (14.03.96)

(54) Title: FUMONISIN-DETOXIFYING ENZYMES

(57) Abstract

Methods for identifying organisms capable of degrading fumonisin. Fumonisin can be incorporated into culture medium for selection of organisms resistant to fumonisin and/or capable of growing on fumonisin as a sole carbon source. Using this method, several organisms have been identified. These organisms can be used to isolate the enzyme and the gene responsible for conferring fumonisin-resistance. The gene can be cloned and inserted into a suitable expression vector so that the protein can be further characterized. Additionally, the DNA encoding for fumonisin-resistance can be used to transform plant cells normally susceptible to *Fusarium* or other toxin-producing fungus infection. Plants can be regenerated from the transformed plant cells. In this way, a transgenic plant can be produced with the capability of degrading fumonisin, as well as with the capability of producing the degrading enzymes. Methods for detoxification in grain processing and in animal feed and rumen microbes are also disclosed.

ACTAGTGGAT CATTCACATG GCTGCGGAC TGGCGCGCG ATAGTCCTTG 1  
CGATGCTGCG GAGATATAGC GTGCGAGTG GAGGATATG AGATGCGGG 51  
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## INTERNATIONAL SEARCH REPORT

International Application No

PC1/US 95/10284

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/55 A01N63/02 C12N15/82 A01H5/00 C12N1/21

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A01N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ARCH. ENVIRON. CONTAM. TOXICOL., vol. 23, 1992 pages 464-467, R.F. VESONDER ET AL.; 'Comparative phytotoxicity of the fumonisins, AAL-toxin and yeast sphingolipids in Lemna minor L. (Duckweed)' cited in the application see abstract and Figure 1. ---	1
A	BIODEGRADATION, vol. 4, 1993 pages 171-178, P.V. BUNZ ET AL.; 'Purification of two isofunctional hydrolases (EC 3.7.1.8) in the degradative pathway for dibenzofuran in Sphingomonas sp. strain RW1' see abstract. ---	1

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Date of the actual completion of the international search

23 January 1996

Date of mailing of the international search report

07.02.96

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## INTERNATIONAL SEARCH REPORT

Int. Appl. No.  
PCT/US 95/10284

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>J. BIOL. CHEM., vol. 267, 1992 pages 18814-18820, J.P. DUVICK ET AL.; 'Purification and characterization of a novel antimicrobial peptide from maize (Zea mays L.) kernels' see abstract.</p> <p>-----</p>	1